Faculty of Science
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# Influence of tuna penning activities on soft bottom macrofaunal assemblages

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**ABSTRACT**

Aquaculture is an important food-producing industry that has often been criticised because of its potential adverse influence on water quality and benthic habitats present in the vicinity of a fish farm. A lucrative sector of the aquaculture industry is Atlantic Bluefin Tuna (ABT) ranching. The main source of pollution of the benthic environment at tuna farms is the uneaten feed-fish which accumulates on the seabed below the tuna pens, but the potential influence of ABT farming is expected to differ from those of other fish farm types such as those rearing sea bass and sea bream, because of the use of feed-fish instead of formulated feed and the large size of the farmed fish. Furthermore, differences in the characteristics of the tuna farms and of the receiving environment may result in varying levels of impact, if present. The present study investigated the influence of tuna penning activities on macrofaunal assemblages of the soft sediment habitat present in the vicinity of the fish pens. Grab samples for sediment physico-chemical attributes; namely mean sediment grain size (MSGS), and percent organic carbon content (POCC) and percent organic nitrogen content (PONC) in the sediment; and for macrofaunal studies, were collected from three tuna farms located c. 1 km off the northeastern to southeastern coast of Malta, at incremental distances from the sea cages (i.e., c. 0 m, 100 m, 1 km, and 2 km away) before initiation of the farming activities, and thereafter at six-monthly or annual intervals, over a period of ten years.

The following study aspects were considered: (i) influence of the northeastern farm during its first year of operation on benthic habitat; (ii) use of polychaete, mollusc, amphipod and decapod taxocenes as indicators of the influence of ABT penning on macrobenthic assemblages; (iii) differences in the magnitude and spatial extent of influence of the three ABT farms that differed in size, stocking density, and location, on benthic habitat; (iv) spatial pattern in attributes of the macrofaunal assemblages present in the vicinity of a farm with incremental distance from the tuna pens; (v) suitability of benthic biotic indices (BBIs) AMBI, BENTIX, BOPA, BOPA-Fish farming (BOPA-FF) and M-AMBI, for monitoring the environmental impact of tuna farming; and (vi) temporal patterns in macrofaunal assemblages in the vicinity of three tuna farms over a ten-year period.

Results from the study of the northeastern farm during its first year of operation indicated significantly elevated sediment POCC and PONC, and (albeit not significantly) higher abundance of capitellid polychaetes in the vicinity of the tuna cages, where uneaten feed-fish had accumulated on the seabed. The changes in benthic habitat were conspicuous in autumn towards the end of the tuna penning season, but some benthic recovery was observed after the fallow period. Of the considered taxoenes, polychaetes and amphipods appeared to be good benthic biotic indicators of the impact of tuna penning on macroinvertebrate assemblages. Results from the third study aspect indicated a higher magnitude of influence at the northeastern farm - the largest farm in terms of holding capacity - compared with the two southeastern farms, but a wider spatial extent of impact (1-2 km) was evident at one of the southeastern tuna farms. The spatial pattern in benthic macrofaunal assemblages was characterised by a high impact area directly below the cages, while a significant peak in diversity 100 m away from the cages was observed at only one of the investigated tuna farms. Of the considered BBIs, the BOPA-FF and M-AMBI indices appeared more sensitive to the environmental influence of tuna penning, but variation in Ecological Quality Status (EQS) assignment among BBIs showed the importance of including multivariate data analyses that are traditionally used in aquaculture environmental impact monitoring studies. Results from the sixth study aspect showed that the benthic EQS changed from 'Bad' and 'Poor' to 'Good'/'High' categorisations at the northeastern farm after the first years of operation, but 'Moderate' EQS at the two southeastern farms towards the end of the study period was indicative of a 'press' disturbance. It was concluded that the seasonal nature of ABT penning and often offshore location of the farms, together with reduction of feed wastage, can mitigate the potential adverse benthic influence of these activities, while multiple tuna farms located close to one another result in added loading on the marine environment, hence highlighting the importance of good spatial planning for coastal aquaculture activities. The high spatio-temporal variation in the influence of tuna penning on benthic macrofaunal assemblages in the vicinity of a farm showed the importance of including multiple impacted and reference areas, as well as replicated sampling times in environmental monitoring of tuna farms. The overall findings are discussed in light of: (i) current knowledge on the influence of aquaculture, in particular ABT ranching, on soft bottom macrofauna present in the vicinity of the activity; and (ii) implications for

environmental monitoring and mitigation strategies of tuna penning activities in the Mediterranean, and, in a more local context, the Maltese Islands. Finally, proposals are made for potential further research on aspects of the environmental effects of tuna penning.

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## ABBREVIATIONS

| | |
|---|---|
| ABT | Atlantic Bluefin Tuna |

### MANAGEMENT, LEGISLATION AND MONITORING

| | |
|---|---|
| AMA | Aquaculture Management Area |
| AZA | Allocated Zone for Aquaculture |
| AZE | Allowed Zone of Effects |
| IMTA | Integrated Multi-Trophic Aquaculture |
| DSRSA | Draft Standards for Responsible Salmon Aquaculture |
| MSFD | Marine Strategy Framework Directive |
| PDGF | Policy and Design Guidelines for Fish Farming |
| WFD | Water Framework Directive |
| EIA | Environmental Impact Assessment |
| SEIA | Strategic Environmental Impact Assessment |

### NEWS AGENCIES AND ORGANISATIONS

| | |
|---|---|
| BBC | British Broadcasting Corporation Ltd |
| MT | Malta Today Ltd |
| ERA | Environment and Resources Authority |
| MEPA | Malta Environment and Planning Authority |
| MT NSO | Malta National Statistics Office |
| MESDC | Ministry for the Environment, Sustainable Development and Climate Change |
| EC | European Commission |
| EU | European Union |

| | |
|---|---|
| FAO | Food and Agriculture Organisation of the United Nations |
| FOESA | Spanish Aquaculture Observatory Foundation |
| GESAMP | Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection |
| ICCAT | International Commission for the Conservation of Atlantic Tunas |
| IUCN | International Union for the Conservation of Nature |

MALTESE TUNA FARMS

| | |
|---|---|
| FMG | Farm in between Malta and Gozo |
| NEF | Northeastern Farm |
| SEF 1 | Southeastern ‘Farm 1’ |
| SEF 2 | Southeastern ‘Farm 2’ |

SAMPLING AND EXPERIMENTAL DESIGN

| | |
|---|---|
| Be | Before |
| Af | After |
| BA | Before/After |
| Ti | Time |
| | |
| Aut | Autumn |
| Spr | Spring |
| | |
| Ar | Area |
| Di | Distance |
| Lo | Location |
| Pl | Plot |
| Si | Site |
| C1 | ‘Control 1’ Plot |
| C2 | ‘Control 2’ Plot |
| Co | Control Plots |

| | |
|---|---|
| F | Farm Plot |
| I, Im | Impacted Plot |
| Re | Reference Plot |

MACROINVERTEBRATE TAXA

| | |
|---|---|
| Biv | Bivalvia |
| Cru | Crustacea |
| Ech | Echinodermata |
| Gas | Gastropoda |
| Pol | Polychaeta |
| Ppc | Polyplacophora |
| Sca | Scaphopoda |
| Ter | Terebellidae |

| | |
|---|---|
| Amp | Ampeliscidae |
| Cap | Capitellidae |
| Capr | Caprellidae |
| Dor | Dorvilleidae |
| Fla | Flabelligeridae |
| Gly | Glyceridae |
| Hes | Hesionidae |
| Lep | Leptocardii |
| Lys | Lysianassidae |
| Mac | Mactridae |
| Mae | Maeridae |
| Mal | Maldanidae |
| Nuc | Nuculanidae |
| Par | Paraonidae |
| Phi | Philantidae |
| Pho | Photidae |
| Phox | Phoxocephalidae |
| Phy | Phyllodocidae |

| | |
|---|---|
| Sab | Sabellidae |
| Sip | Sipuncula |
| Syl | Syllidae |
| Uro | Urothoidae |

BENTHIC BIOTIC INDICES

| | |
|---|---|
| A | Total Abundance |
| B | Biomass |
| H', ShW | Shannon-Wiener diversity |
| NFa | Total Number of Families |
| NI | Number of Individuals |
| S | Species Richness |
| P-R | Pearson-Rosenberg |

| | |
|---|---|
| AMBI | Azti Marine Biotic Index |
| BBI | Benthic Biotic Index |
| BOPA | Benthic Opportunistic Polychaetes and Amphipods Index |
| BOPA-FF | BOPA-Fish Farming Index |
| M-AMBI | Mulitvariate-Atzi Marine Biotic Index |
| P/A | Polychaete/Amphipod Ratio |

| | |
|---|---|
| EQS | Ecological Quality Status |
| EnQS | Environmental Quality Standards |

| | |
|---|---|
| EG | Ecological Group |
| GI | Sensitive Taxa |
| GII | Indifferent Taxa |
| GIII | Tolerant Taxa |
| GIV | Second-Order Opportunistic Taxa |
| GV | First-Order Opportunistic Taxa |

| | |
|---|---|
| $f_A$ | Frequency of Amphipods (excluding *Jassa*) |

| | |
|---|---|
| $f_P$ | Frequency of Tolerant Polychaetes |

| | |
|---|---|
| B EQS | 'Bad' Ecological Quality Status |
| P EQS | 'Poor' Ecological Quality Status |
| M EQS | 'Moderate' Ecological Quality Status |
| G EQS | 'Good' Ecological Quality Status |
| H EQS | 'High' Ecological Quality Status |

SEDIMENT PHYSICO-CHEMICAL ATTRIBUTES

| | |
|---|---|
| FF | Feed-fish |
| FFBC | Feed-fish bone content (%) |
| MSGS | Mean Sediment Grain Size (phi) |
| POCC | Percent Organic Carbon Content |
| PONC | Percent Organic Nitrogen Content |

STATISTICAL ANALYSES AND RESULTS

| | |
|---|---|
| SE | Standard Error |
| α | Level of Significance |

| | |
|---|---|
| BIOENV | Biota and/or Environment Matching |
| PCO | Principal Coordinate Analysis |
| PERMANOVA | Permutational Analysis of Variance |
| PERMDISP | Permutational Multivariate Dispersion |
| SIMPER | Similarity Percentages of Species Contributions |

| | |
|---|---|
| df | Degrees of Freedom |
| p-value | Calculated probability |
| p(MC) | Monte Carlo p-value |
| p(PERM) | Permutational p-value |
| p(Tables) | p-value from Tables |
| Sqr Rt Var | Square Root Estimate of Component of Variation |

| | |
|---|---|
| RES | Residual Variation |
| | |
| * | p-value < 0.05 |
| ** | p-value < 0.01 |
| *** | p-value < 0.001 |
| **** | p-value < 0.0001 |
| ns | Not Significant |
| | |
| AsC | Assemblage Composition |
| Av Abund | Average Abundance |
| Avg Dissim (%) | Average Dissimilarity (%) |
| Avg Sim (%) | Average Similarity (%) |
| Best Exp Var | Best Explanatory Variable |
| Contrib (%) | Contribution (%) |
| Cum Contrib (%) | Cumulative Contribution (%) |
| Dep Var | Dependent Variable |
| ρ-value | Spearman's rank correlation coefficient |
| | |
| PC1 | First Principal Coordinates Axis |

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Data on attributes of the benthic macrofaunal assemblages and sediment quality (i.e. sediment weight/weight feed-fish bone content, mean sediment grain size, and percent organic carbon content and percent organic nitrogen content in the sediment), and on sea current direction and velocity; that are used in the present research; were collected by the Maltese independent consultants Ecoserv Ltd respectively between 2000 and 2009, and between 2010 and 2017, as part of the environmental monitoring programme described below in Section 1.3.3 for the three Maltese tuna farms included in the present study. I am deeply grateful to Ecoserv Ltd for rendering these datasets available for my doctoral studies.

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# CHAPTER 1
# GENERAL INTRODUCTION

## 1.1 Aquaculture

### 1.1.1 Aquaculture in food production

Aquaculture contributes to an important and rapidly expanding food-producing sector (Food and Agriculture Organization of the United Nations [FAO], 2010; Spanish Aquaculture Observatory Foundation [FOESA], 2010). Globally, aquaculture production has increased at an average rate of 8.8% per year since the 1980s and 1990s, after which it continued to increase in the 2000s, albeit at a slower rate (FAO, 2012). Aquaculture products exceeded capture fisheries products for the first time in 2014, comprising 73.8 million t of aquatic animals in 2014 worth about 147.516 billion Euro and aimed primarily for human consumption (FAO, 2016). Such an average annual growth rate is the fastest of all livestock agricultural production sectors, and is more than double that of poultry, pigs and cattle (Barazi-Yeroulanos, 2010). At present, the greater part of fish intended for worldwide human consumption is sourced from aquaculture, and the current production level has exceeded population growth, resulting in a higher per capita fish supply in most regions (FAO, 2016). Aquaculture is therefore set to be a very important contributor in the food production sector.

### 1.1.2 Concerns on aquaculture and its environmental influence

Aquaculture has raised concerns (e.g. Allsopp, Johnston, & Santillo, 2008; Esmark & Cripps, 2003) because of its potential adverse influence on the environment, including ones resulting from the several impacts of escapees, attraction of populations of wild fish around offshore fish cages, use of fish oil and fish meal in feeds, and deterioration of water quality and of biotic assemblages and habitats (Hargrave *et al*., 1997; Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection [GESAMP], 1990; Milewski, 2001; Wu, 1995). Aquaculture also raises several socio-economic concerns due to conflict with other competing coastal resource users. Net-pens occupy space in coastal areas, impact the aesthetics of the coastal zone, are a navigational hazard, and create problems due to smells and pollution (Ottolenghi, Silvestri, Giordano, Lovatelli, & New, 2004).

The rapid expansion of the activity, which increases the demand on coastal resources (Giles, 2008), appears to have aggravated the situation. As a result, several non-governmental entities are currently engaged in efforts aimed at identifying and advocating measures to reduce or possibly eliminate the undesirable environmental effects of aquaculture and to make it more sustainable (FAO, 2010; FOESA, 2010; International Union for Conservation of Nature [IUCN], 2009a, 2009b, 2009c). Such measures include greater attention given to site selection and management (IUCN, 2009a, 2009b), and identification of suitable indicators (Giles, 2008; FOESA, 2010) and strategies for adopting effective monitoring programmes (Fernandes *et al*., 2001). Most countries are moving towards the identification of appropriate sites for use as "Allocated Zones for Aquaculture" (AZAs), where marine aquaculture is prioritised over other competing uses of the coastal zone, and through adoption of several measures to reduce environmental impacts; for example, by using an ecosystem-based management approach (FAO, 2013; Sanchez-Jerez *et al*., 2016). However, successful implementation of such measures is highly effected by the availability of information concerning aquaculture and environment interactions, on the basis of which coastal planners and managers may make informed decisions and formulate appropriate coastal management plans. Effective environmental impact assessments and monitoring of aquaculture activities are therefore deemed very important (Wu, 1995).

The interactions of aquaculture with the environment vary with site characteristics such as water depth and bottom currents, and with management practices such as the farmed species, feed type and stocking density (Wu, 1995). A substantial amount of organic matter input to aquaculture is lost to the environment as waste, mainly via uneaten feed and faeces, while chemicals such as pesticides and medications and new genetic strains and pathogens may also be introduced to the environment (Wu, 1995). Most of the environmental effects of aquaculture are expected to be adverse, however, the magnitude of influence varies (Karakassis & Dror, 2011); some effects result from poor management practices and may be easily reduced, but ones resulting from uneaten feed and faeces are intrinsic to fish farming and difficult to avoid (Karakassis & Dror, 2011).

Price, Black, Hargrave and Morris (2015) provide a general review on the effect of modern aquaculture activities on water quality and primary production. An overview

of the potential influences of aquaculture on the marine ecosystem is given in Table 1.1, however, it should be noted that the main impact is on benthic habitats (Wu, 1995).

### 1.1.3 Aquaculture influence on benthic habitat

Uneaten feed and fish faeces that accumulate on the seabed below fish cages form a decomposing mass of organic matter that results in enhanced microbial activity (Giles, 2008). Increased microbial activity increases the consumption of oxygen (the electron acceptor in aerobic respiration) from porewater, where it travels slowly from oxygenated water on the surface sediment via diffusion, potentially leading to low levels of porewater oxygen and hypoxic or anoxic sediment conditions (Sanz-Làzaro & Marin, 2011). In such conditions, anaerobic bacteria that use other electron acceptors, mainly sulfate (found very abundantly in sea water), take over the role of organic matter mineralisation and produce high sulfide concentrations in organically enriched sediments (Sanz-Làzaro & Marin, 2011). Once sulfate is depleted, methanogensis takes place, producing methane (Sanz- Làzaro & Marin, 2011). Suspension-feeding crustacean infauna are the first to be affected adversely by such conditions, and are replaced by a high abundance of opportunist, deposit-feeding taxa, tolerant to pollution, which in turn results in reduced benthic diversity (Giles, 2008). The level of adverse influence on benthic habitat varies with distance from the farm (Kalantzi & Karakassis, 2006) as well as with time following cessation of an aquaculture activity (e.g. Macleod, Moltschaniwskyj, Crawford, & Forbes, 2007; Macleod, Moltschaniwskyj, & Crawford, 2008; but see also Karakassis, Hatziyanni, Tsapakis, & Plaiti, 1999; Sanz-Làzaro & Marin, 2006), as described by the Pearson-Rosenberg (P-R) (1978) model and modified in later works for macroinvertebrate assemblages along an organic enrichment gradient (Sanz-Làzaro & Marin, 2011) (Figure 1.1).

The influence on benthic habitat is generally limited to the immediate vicinity of the cages (e.g. Karakassis, Tsapakis, Hatziyanni, Papdopoulou, & Plaiti, 2000; Karakassis, 2001; Tomassetti *et al*., 2016), but may extend from several meters to hundreds of kilometres from the farm (Fernandez-Gonzalez, Aguado-Giménez, Gairin, & Sanchez-Jerez, 2013). While particulate organic matter settles below fish cages located in low current velocities, it is deposited further away in stronger currents via re-suspension of

**Table 1.1** Potential influences of aquaculture on the marine ecosystem after Milewski (2001).

| | |
|---|---|
| | Physical presence of cages |
| Attraction of wild fish | Persistent large aggregations of wild fish at fish farms are vulnerable to pathogen transfer from farmed fish and to hidden fishing practices from within the leased area (e.g. Bacher & Gordoa, 2015; Dempster *et al*., 2009). |
| Escapement of farmed fish | Escapees may introduce pathogens to wild populations of fish, become established as introduced species, alter indigenous gene pools, and result in a decline in endangered fish species via predation and competition (Milewski, 2001). |
| | Feeding of farmed fish |
| Use of bait fish | The use of declining wild populations of small pelagic fish from offshore marine systems (where they act as primary food sources for top predators), to produce fish oil and fish meal, may have inter-ecosystem impacts (Milewski, 2001) |
| Particulate waste | Accumulation of uneaten feed and faeces on the seabed below fish cages may lead to anaerobic sediment conditions (Giles, 2008), damaging maerl beds (e.g. Hall-Spencer, White, Gillespie, Gillham, & Foggo, 2006; Sanz-Lázaro, Belando, Marín-Guirao, Navarrete-Mier, & Marín, 2011) and sea-grass meadows (e.g. Pergent-Martini, Boudouresque, Pasqualini, & Pergent, 2006), while macroinvertebrate assemblages become dominated by a few opportunistic species, leading to reduced benthic diversity (Giles, 2008). |
| Dissolved waste | Additive effects of nutrient loading from multiple farms may lead to far-field eutrophication and formation of harmful algal blooms with toxic or harmful effects on fish and invertebrates (Price *et al*., 2015). |
| | Use of chemicals |
| Antibiotics | Antibiotics are released in active form from uneaten feed and faeces, accumulating in high levels in sediment and invertebrates, and in wild fish potentially intended for human consumption (Milewski, 2001). |
| Pesticides | Similar accumulation of the active ingredients and carrier chemicals of pesticides and anti-fouling agents may have toxic or sub-lethal effects in invertebrates and fish (Milewski, 2001). |

the surface sediment (Kutti, Ervik, & Hansen, 2007a), resulting in a reduced level of benthic influence (e.g. Maldonado *et al*. 2005; Moraitis, Papageorgiou, Dimitriou, Petrou, & Karakassis, 2013; Puhr, Pikelj, & Fiket, 2017; Vezzulli *et al*., 2008; but see also Hall-Spencer *et al*., 2006; Lee, Bailey-Brock, & McGurr, 2006; Valdemarsen, Bannister, Hansen, Holmer, & Ervik, 2012) but potentially wider spatial footprints (Hall-Spencer *et al*., 2006).

Macroinvertebrate assemblages are more resilient to aquaculture influence in sediments that have a low percentage of fines fraction (Borja, Dauer, Elliott, & Simenstad, 2010a; Papageorgiou, Kalantzi, & Karakassis, 2010) and in sites that have

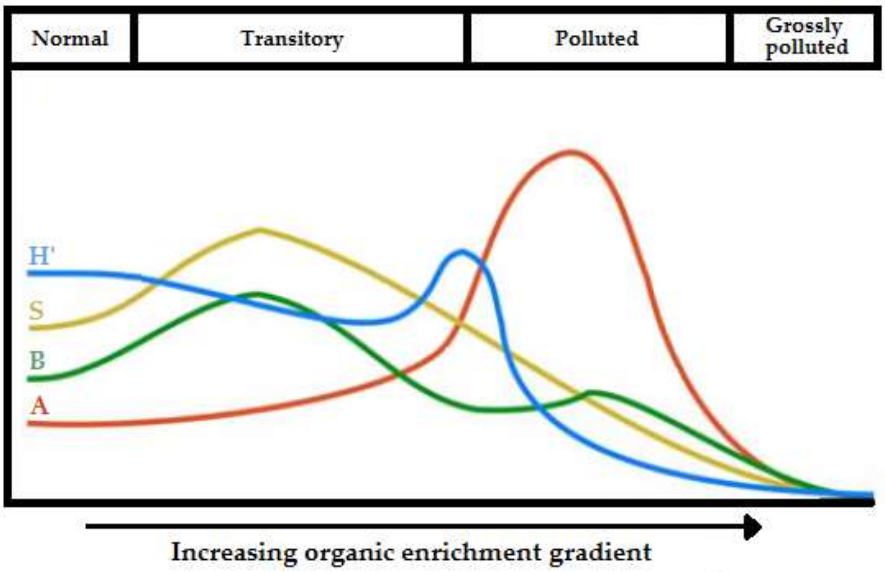


**Figure 1.1** The P-R model for macrofaunal assemblages along an organic enrichment gradient, showing the theoretical succession from the 'normal' to the 'transitory', 'polluted', and 'grossly polluted' benthic stages (Sanz-Làzaro & Marin, 2011). The initial increase in total abundance (A), biomass (B), richness (S), and diversity (H') with increasing organic loading, peaks at intermediate levels of organic enrichment and decreases as the sediment becomes azoic (Sanz-Làzaro & Marin, 2011). The peak in diversity defines the ecotone point and is followed by the polluted zone, in which a peak in the total abundance is recorded due to a peak in opportunistic species (Sanz-Làzaro & Marin, 2011). Reproduced from Sanz-Làzaro and Marin (2011).

naturally high sedimentation regimes that determine the biotic community structure in terms of species tolerance to enrichment (Macleod *et al*., 2007).

The periodic abandonment of cage sites (fallowing) is a sustainable aquaculture practice that allows recovery of the benthic habitat to take place between production cycles (Fernandes *et al*., 2001), and prevents overloading and defaunation of the sediment (Macleod, Moltschaniwskyj, & Crawford, 2006; Macleod *et al*., 2007). However, long recovery times (between 2 to > 7 years) are reported for benthic assemblages following cessation of aquaculture activities (Borja *et al*., 2010a), indicating that a benthic community may return to the pre-fallowed state as soon as production is resumed, even after a two-year fallow period (Pereira, Black, Mclusky, & Nickell, 2004).

## 1.2 Tuna penning

### 1.2.1 Tuna penning: a lucrative sector of aquaculture

A lucrative sector of aquaculture is the farming of Atlantic Bluefin Tuna (ABT) *Thunnus thynnus thynnus* Linnaeus 1758. Good quality, fatty, ABT has a very high culinary value and constituted 8% of the total fish exports in 2010 (FAO, 2012): a single bluefin tuna of 222 kg weight fetched the record price of 115 million yen (961,000 Euros) at the first auction of 2013 of Japan's Tsukiji fish market (British Broadcasting Corporation [BBC], 2016). Tuna penning is considered by many as not being "true" aquaculture but a "capture based" variant of the activity, since the seed is harvested from the wild. Each year, during the period May to July, tuna is harvested from the wild using purse-seine vessels, and the fish are then transferred to offshore floating cages (Camilleri, 2017) where they are overfed with fresh fish (sardine, mackerel and other clupeid fishes) and molluscs (including squid) (see Aguado, Martinez, & Garcia-Garcia, 2004; Vita & Marin, 2007). The overfeeding regime is aimed at reaching elevated lipid contents in the fish (Aguado-Giménez, García-García, Hernández-Lorente, & Cerezo-Valverde, 2006). During the period October to January, the fish are harvested and exported to Japan (Camilleri, 2017).

### 1.2.2 Development of tuna penning activities

The general development of the tuna penning industry is well described by Miyake (2005, 2007). Large-scale ABT penning started in Canada in the 1980s, and in the 1990s the activity spread to Spain and throughout the Mediterranean (Miyake, Guillotreau, Sun, & Ishimura, 2010). It now constitutes a large sector within the fish farming industry (Ottolenghi *et al*., 2004), with the main Mediterranean tuna farms being located in Italy, Malta and Spain (International Commission for the Conservation of Atlantic Tunas [ICCAT], 2011). Tuna farms are also present in Croatia, Turkey, Cyprus, Greece, Tunisia, Libya, Portugal and Morocco (ICCAT, 2011). The number of tuna farms and their individual total capacity in each Mediterranean country where the activity is carried out must be registered with the ICCAT. Currently, fifty five Mediterranean tuna farms, having a total capacity of 59,462 t, are registered with the ICCAT. Of these, eight, having a total capacity of

12,300 t, are located in Malta (ICCAT, 2011), but are not used to their maximum capacity.

Aquaculture in the Maltese Islands started on an industrial scale in the early 1990s, with nearshore farming of sea bass and sea bream (Holmer, Hansen, Karakassis, Borg, & Schembri, 2008). Between 1990 and 2000, local aquaculture production increased from 100 t to 2000 t. The eight farms that contributed to the activity were located in shallow sheltered waters, in the vicinity of *Posidonia oceanica* meadows (Holmer *et al*., 2008). However, strong competition from mainland Europe, as well as high operational costs, led to a general decline in sea bass and sea bream farming in Malta during the late 1990s, with the result that, in 2000, tuna penning, which was relatively new and highly successful in Europe, was introduced and became the main aquaculture activity locally (Holmer *et al*., 2008). Malta quickly became one of the top exporters of farmed ABT in the Mediterranean; production increased steadily from 300 t in the early 2000s to 3,000 t by 2005 (Holmer *et al*., 2008). In 2006, tuna penning was also started at an offshore AZA located some 6 km off the southeastern coast of Malta (Figure 1.2) (Holmer *et al*., 2008).

In the early 2000s, three tuna farms were deployed some 1 km off the coast of Malta (Figure 1.2): one off the northeastern coast, having eight cages with a maximum holding capacity of 2,500 t; and two smaller farms, one having four cages and the other having three cages (maximum holding capacity of 1500 t each), both of which were located off the southeastern coast (ICCAT, 2011). A fourth tuna farm (maximum holding capacity of 800 t) was located in the channel between Malta and Comino (ICCAT, 2011) (Figure 1.2). The three tuna farms that were located 1 km offshore were sited in waters some 50 m deep characterized by strong bottom currents (Holmer *et al*., 2008). Recently (in May2017), these three farms, together with that located in the Malta – Comino Channel, had their permits revoked by the local Environment and Resources Authority (ERA) in 2016, and were translocated further offshore; the two farms off the southeastern coast were moved to the offshore AZA, which already accommodates four other tuna farms (Figure 1.2) (ICCAT, 2011) in waters that are some 90 m deep (Camilleri, 2017), while the other two farms were translocated to a site located 6 km off the northeastern coast. Of the four farms that were already present in the offshore AZA (since 2006), one farm has a maximum holding capacity of 3000

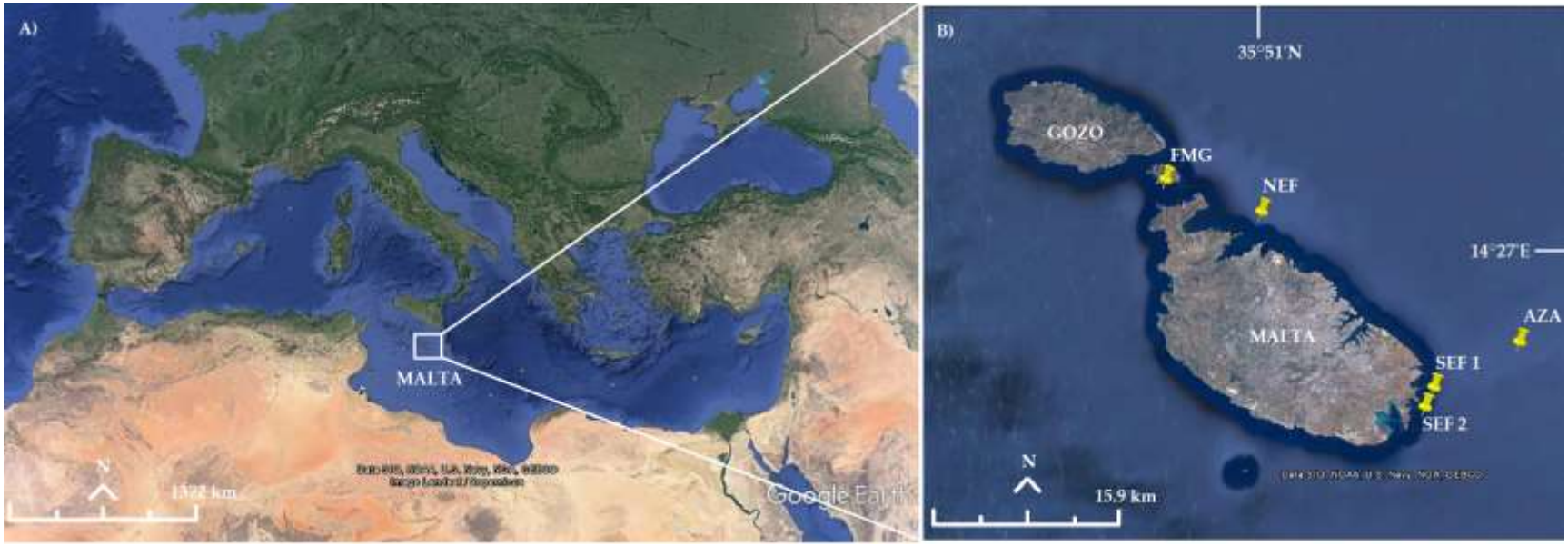


**Figure 1.2** Satellite imagery reproduced from Google Earth (Data SIO, NOAA, US Navy, USA, GEBCO) showing: (a) the location of the Maltese Islands in the Mediterranean Sea, and (b) the location of the Maltese tuna farms: one in the channel between Malta and Gozo (FMG), four in the offshore AZA, and three farms; a northeastern farm (NEF) and two southeastern farms (SEF 1 and SEF 2); sited circa 1 km off the coast of Malta (Camilleri, 2017). Another offshore AZA is planned for the northeastern coast of Malta (Camilleri, 2017).

t, while the other three farms are smaller (maximum holding capacities of 1,500 t, 750 t, and 750 t) (ICCAT, 2011).

Maltese tuna farms utilize cages having a diameter of some 50 m and a height of around 25 m (Holmer *et al*., 2008), while a few 90 m diameter tuna cages have been in use since 2003 (Camilleri, 2017). In 2014, Maltese tuna farms produced some 5,451 t of ABT worth 81.462 million Euro (Malta National Statistics Office [MT NSO], 2015).

### 1.2.3 Environmental impact of tuna penning

As with most other aquaculture operations, uneaten feed-fish and excreted waste are the main source of pollution in tuna penning. The uneaten fish that accumulate on the seabed below the tuna-pens (Aguado, Martinez, & Garcia-Garcia, 2004; Aguado-Giménez *et al*., 2006; Borg & Schembri, 2005; Vita & Marin, 2007; Vita *et al*., 2004a) lead to adverse effects on the species composition and structure of benthic assemblages present in their vicinity (Borg & Schembri, 2005; Vezzulli *et al*., 2008; Vita & Marin, 2007; Vita *et al*., 2004a). Potential adverse environmental effects may be reduced or eliminated when the tuna pens are located well offshore in a high energy environment (Aksu, Kaymakçı-Basaran, & Egemen, 2016; Maldonado, Carmona, Echeverría, & Riesgo, 2005; Moraitis *et al*., 2013). Although ABT is farmed under high stocking densities, which entail high feed input, these vary among different farms and even between different cages within the same farm. As a result, one would expect large differences in the level of adverse influence, where present. Furthermore, because of the particular characteristics of the activity, namely use of feed-fish instead of processed feed, as well as the large size of the fish, the potential adverse influence of tuna penning is expected to differ from that of other fish farming activities such as salmon, sea bream and sea bass farming.

In the Mediterranean, studies that have assessed the amount of organic waste generated by ABT farming in SE Spain report a gross waste output at tuna farms which is 7 times higher than that produced by Mediterranean sea bass or sea bream farms (Aguado-Giménez *et al*., 2006). Furthermore, production of particulate waste has been estimated as being of the order of circa 9 mg phosphorus and 6 mg nitrogen per kg of fish per

day (Vita *et al*., 2004a). Other studies in Australia assessed nutrient leaching from feed and faeces generated at southern bluefin tuna (*Thunnus maccoyii*) farms, and reported high losses of nitrogen to the environment; circa 90 % of which were lost as dissolved wastes (Fernandes, Angove, Sedawie, & Cheshire, 2007a; Fernandes, Lauer, Cheshire, & Angove, 2007b).

Studies assessing the influence of dissolved wastes from ABT farms on nutrient levels in the surrounding seawater reported enhanced nutrient concentrations in the water column below fish cages in Croatia (Matijević, Kušpilić, & Barić, 2006) and the presence of organic waste in the water column up to 1 km away from cages in Sicily (Italy) (Vizzini & Mazzola, 2012). On the other hand, no significant effects on nutrient levels in the surrounding seawater have been reported for ABT farms located in exposed sites in Turkey (Aksu, Kaymakçi-Başaran, & Egemen, 2010; Aksu *et al*., 2016) and on the taxonomic abundance of zooplankton in southwest Italy (Vezzulli *et al*., 2008).

Other works assessed changes in the physico-chemical properties of sediments in the vicinity of ABT farms in SE Spain, where low silt-clay fraction, and high organic content, acid-volatile sulfur, and total ammonical nitrogen in sediments were reported in sediments below the fish cages (Marin *et al*., 2007; Vita & Marin, 2007). Vezzulli *et al*. (2008) reported low sediment redox potential below ABT cages in SW Italy, while Dal Zotto, Santulli, Simonini, and Todaro (2016) reported high organic content and sulfides levels in the sediment below ABT farms in Sicily. Other workers assessed changes in the physico-chemical properties of sediments in the vicinity of ABT farms in Croatia, where negative sediment redox-potential (Matijević *et al*., 2006) and high organic carbon, total nitrogen (Matijević *et al.*, 2006), and total phosphorus (Matijević *et al*., 2006; Matijević, Kušpilić, Kljaković-Gašpić, & Bogner, 2008), were reported in the sediment below the cages. On the other hand, Aksu *et al*. (2016) reported no significant influence of tuna penning activities on sediment organic carbon content in Turkey, which was attributed to the controlled feeding regime and exposed location of the farm.

Studies by Croatian workers have also shown the effect of ABT farms as aggregation devices for wild fish assemblages particularly ones belonging to the families Sparidae

and Belonidae (Šegvić Bubić, Grubišić, Tičina, & Katavić, 2011), as well as negative effects of the farming activity on the shoot density of *Posidonia oceanica* meadows; the latter resulting from the organic waste input which promotes increased epiphyte growth on the seagrass leaves (Kružić, Vojvodić, & Bura-Nakić, 2014). A study by Hospido and Tyedmers (2005) highlighted the indirect effects of the Spanish ABT penning industry via the use of diesel fuel during fishing and post-harvest transport of tuna, while a study by Forrestal, Coll, Die, and Christensen (2012) that uses food-web models focused on the increasing ecosystem influence of ABT farming in Spain that results from removal of tuna and small pelagic feed-fish from top and intermediate trophic levels, respectively.

A few studies assessed the influence of ABT ranching on benthic fauna; for example, Dal Zotto *et al*. (2016) reported a significant increase in meiofaunal abundance, and decreased diversity of kinorhynchs, in the vicinity of ABT pens in Sicily (Italy). Studies on the influence of ABT ranching on macroinvertebrate assemblages in Spain reported a spatial gradient of stressed assemblages, with a high impact radius that is generally limited to the immediate vicinity of the cages (Marin *et al*., 2007; Vita & Marin, 2007). One of these same studies noted an incomplete recovery of the benthic biotic assemblages following a 6-month fallow period (Vita & Marin, 2007). Jahani *et al*. (2012) reported low macrofaunal abundance, biomass and diversity below ABT cages in Greece, while Moraitis *et al*. (2013) found no significant influence on macrofaunal assemblages in the vicinity of ABT farms in Cyprus, but which was attributed mainly to exposed nature of the farm site.

Clearly, numerous studies on the influence of ABT farming on the marine environment are available, yet there is a need for more detailed study on the influence of the activity on benthic habitat given that: (i) no study has compared attibutes of benthic habitat before and after initiation of tuna penning activities; (ii) there is no overall agreement on which biological indicator best signals change in the marine environment under the influence of tuna penning activities; (iii) there is a need to assess for differences in the level and spatial extent of influence of tuna penning between tuna farms that differ in size and other operational aspects; (iv) the studies that assessed spatial patterns in tuna penning influence on benthic habitat examined distances in the range of 100 m from the cages and did not include far-field effects in the range of 1 km and 2 km from the

cages; (v) no study has examined the suitability of macroinvertebrate indices developed under the EU's WFD to monitor the benthic influence of tuna penning activities in the Maltese Islands; and (vi) there is a need to establish temporal patterns in tuna penning influence on benthic habitat to determine whether the activities form a 'pulse'- or 'press'- type of disturbance.

## 1.3 Environmental monitoring

Traditional environmental monitoring of aquaculture activities comprises analyses of benthic physico-chemical and macroinvertebrate data (Aguado-Giménez *et al*., 2007). Macroinvertebrate groups that are known to be good indicators of biological change resulting from fish farm wastes include polychaetes (e.g. Aguado-Giménez *et al*., 2015; Martinez-Garcia *et al*., 2013; Sutherland, Levings, Petersen, Poon, & Piercey, 2007; Tomassetti & Porrello, 2005) and amphipods (e.g. Fernandez-Gonzalez & Sanchez-Jerez, 2011).

### 1.3.1 Environmental monitoring of aquaculture in the Maltese Islands

In Malta, the Environment and Resources Authority (ERA), previously the Malta Environment and Planning Authority (MEPA), grants development permits for proposed aquaculture operations on the condition that a regular environmental monitoring programme is undertaken by independent environmental consultants according to pre-defined operational guidelines for local aquaculture activities (Malta Environment and Planning Authority [MEPA], 2012).

During the initial phase of introduction of tuna penning to Malta, the large scale of the proposed operations raised great public concern, which led the MEPA to amend its Policy and Design Guidelines for Fish Farming (PDGF) in 2001 (see Holmer *et al*., 2008). The amendments set the condition that any proposed aquaculture operations must be at located at least 1 km offshore, in waters exposed to strong sea currents, and located away from *P. oceanica* meadows and other habitats of high ecological importance such as maerl beds (Holmer *et al*., 2008). As a result, the three farms that commenced tuna penning activities in the early 2000s were located in a water depth of some 50 m over bare soft sediment habitat (Holmer *et al*., 2008).

In the 2001 PDGF document, the MEPA also requested regular environmental monitoring to be carried out with respect to water and sediment quality, benthic diversity, gross biological and physical features of the seabed below the tuna pens, and assessments of the state of habitats at important dive sites located at some distance (but not in the immediate vicinity of) the farms (see Holmer *et al*., 2008).

### 1.3.2 'Bare sand' habitat and associated macrobenthic assemblages

Extensive seabed areas with bare soft sediment supporting macrobenthic assemblages that are characteristic of this habitat are present off the northeastern coast of the Maltese Islands down to a depth of around 50 m (Grech Santucci, 2005). The largest seabed areas with this habitat type occur between the *P. oceanica* meadows present in the Infralittoral Zone and the maerl beds present in the Circalittoral Zone. Typically, the sediment that characterises bare soft sediment habitat consists of poorly sorted fine to very fine sand that have low fractions of silt-clay and coarse sediment (Grech Santucci, 2005).

The biota associated with the bare soft sediment habitat is characterised by a high abundance of polychaete and crustacean species, many of which also occur on other mobile detritic habitats, maerl beds and *P. oceanica* meadows (Grech Santucci, 2005). Of the macrobenthic species recorded from the bare sand habitat, *Aspidosiphon muelleri* (Sipuncula), *Urothoe* spp. (Amphipoda), *Apseudes* sp. (Tanaidacea), and *Paraonidae* spp. and *Glyceridae* spp. (Polychaeta) dominate the assemblages, and account for some 25% of the total abundance (Grech Santucci, 2005). Sediment characteristics and the associated macrofaunal assemblages are rather homogenous at the large spatial scale (5 km to 16 km), but at the small spatial scale (1 km) heterogeneous sediments under the influence of bottom currents support more species rich and diverse benthic assemblages. On the other hand, in the case of sediments with decreased physical structure that results from sedimentation events linked to organic effluents, anoxic conditions develop and assemblages are impoverished (Grech Santucci, 2005). Seasonal effects have not been found to have a large influence, overall, on the structure of benthic assemblages (Grech Santucci, 2005).

### 1.3.3 Environmental monitoring of tuna penning in the Maltese Islands

Environmental monitoring of tuna penning activities in the Maltese islands was initiated in 2000 (Holmer *et al*., 2008), and is still ongoing. Prior to initiation of the local tuna penning activities, grab samples of sediment and benthos were collected at each of the three tuna farms that were sited circa 1 km off the northeastern to southeastern coast of Malta from stations below the pens, some 100 m away from the pens (Plot A), and from control sites in the range of 1 - 2 km away (Plots B & C) (Figures 1.3.a, 1.4.a, 1.4.b). The northernmost farm (NEF) deployed four tuna cages, while one of the southeastern farms (southeastern 'Farm 1'; SEF 1) deployed three cages and the other southeastern farm ('Farm 2'; SEF 2) deployed four cages. The designated farm lease areas (Plot A) were: 350 m x 500 m at the NEF (Figure 1.3.a), 550 m x 550 m at the SEF 1 (Figure 1.4.a), and 300 m x 500 m at the SEF 2 (Figure 1.4.b). A summarised description of the three tuna farms is given in Table 1.2.

Tuna penning activities were initiated in May-June 2001 at the NEF and SEF 2, and in May – June 2003 at the SEF 1. Environmental monitoring at the NEF was carried out two times per year prior to initiation of the tuna penning activities (in November 2000 and in March 2001), and again two times per year during the first two years of production (in November 2001 and April 2002, and in January 2003 and April 2003), and on annual basis thereafter in: November 2003, November 2004, November 2005, April 2006, June 2007, May 2008, and April 2009. In early 2001, the existing NEF lease area was extended by 550 m (Plot D) to accommodate four more tuna pens (Figure 1.3.b). Since Plot B was located within the potential influence area of the extended NEF, three new control areas (Plots E, F, and G) were included in the sampling design (Figure 1.3.b), but later (in 2003), Plots F and G (which supported a different benthic habitat type, i.e. maerl interspersed with patches of 'bare sand' habitat) (Figure 1.3.c) were eliminated from the sampling programme, together with Plot B since this plot ended up too close to the tuna penning site and hence deemed to be potentially influenced by the tuna farming activities. Later, in November 2005, plots A and D were fused into a single plot 'AD' measuring some 1000 m x 700 m (Figure 1.3.d).

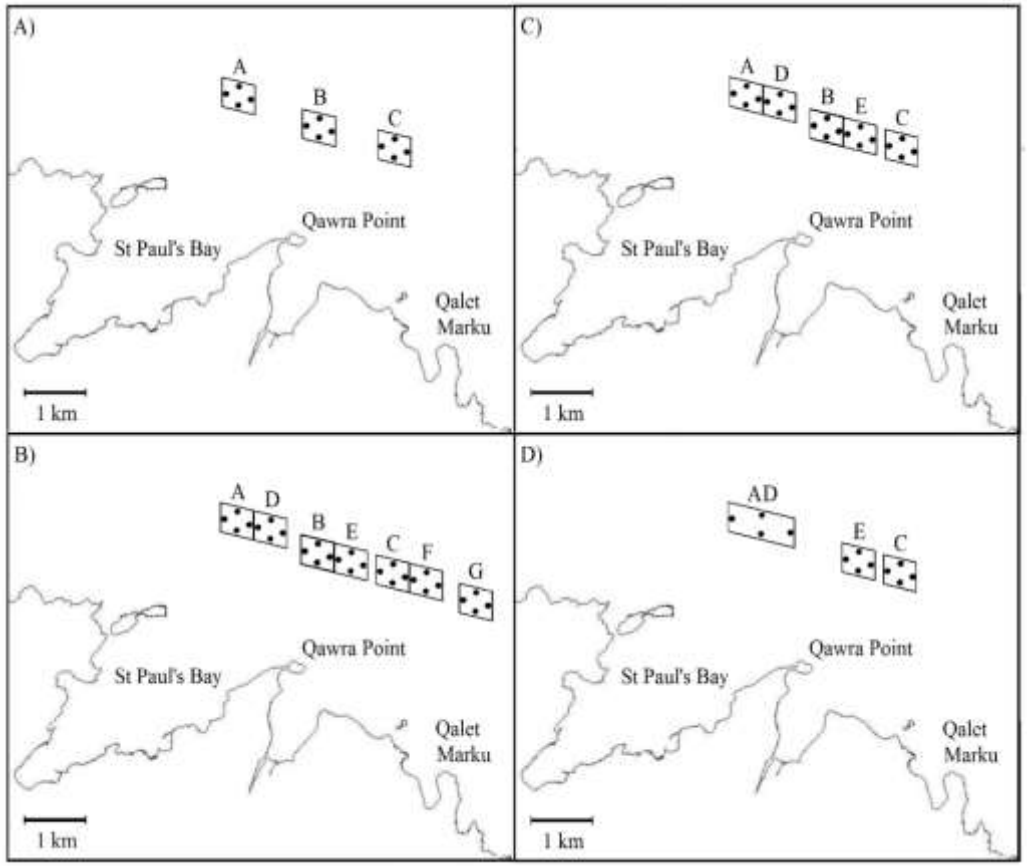


**Figure 1.3** Map of the northeastern study area showing the locations of: (a) Plots A, B, and C that were monitored in November ’00 and March ’01; (b) Plots A, D, B, E, C, F, and G that were monitored in November ’01 to April ’03; (c) Plots A, D, B, E and C that were monitored in November ’03 and November ’04; and (d) Plots AD, E, and C that were monitored in November ’05 to April ’09. At all plots, samples for benthic macrofaunal and sediment physico-chemical studies were collected.

**Table 1.2** Main characteristics of the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1) and southeastern 'Farm 2' (SEF 2), and of the coastal sites where they were located.

| | NEF | SEF 1 | SEF 2 |
|---|---|---|---|
| Water depth (m) | 48-52 | 43-51 | 43-52 |
| Approximate distance from shore (km) | 1 | 1 | 1 |
| Maximum total annual capacity (t) | 2500 | 1500 | 1500 |
| Benthic habitat type | 'Bare sand' | 'Bare sand' | 'Bare sand' |
| Mean sea current direction (º) | 182 $\pm$ 96 | 187 $\pm$ 84 | |
| Mean sea current velocity (ms $^{-1}$) | 0.088 $\pm$ 0.103 | 0.185 $\pm$ 0.175 | |
| Fallow period (months) | 5-6 | 5-6 | 5-6 |

Environmental monitoring of the southeastern farms was carried out on an annual basis in autumn at the SEF 1 (October 2002 to October 2005) (Figure 1.4.a), and in spring at the SEF 2 (June 2001 to June 2003) (Figure 1.4.b), at Plots A, B and C. In June 2004, the existing SEF 2 farm lease area was extended to some 450 m x 500 m with the introduction of four more tuna pens, and a new control plot (Plot D) was accordingly added to the monitoring design (Figure 1.4.c). In June 2006, the cost-benefit ratio of the environmental monitoring programme at the two southeastern farms was eventually maximised by reducing the sampling effort to three common control sites (Figure 1.4.d), and by elimination of the SEF 2 Plot B from the sampling design (Figure 1.4.e).

Direct observation of gross biological and physical features of the sediment below the tuna pens has also been carried out by SCUBA divers, who used underwater videography to record their observations. For this monitoring component, a semi-quantitative index was developed to quantify and compare the amount of uneaten feed-fish that had accumulated on the sediment below different tuna pens (Table 1.3).

Mapping surveys that incorporated underwater videography to monitor the state of health of important benthic habitats such as seagrass beds, as well as of seabed physical and biological characteristics at popular dive sites, have also been undertaken by scientific SCUBA divers. Additionally, water quality surveys were carried out in the immediate vicinity of the tuna pens and at control sites. The monitored water quality attributes include chlorophyll a, dissolved oxygen, salinity, temperature, turbidity, ammonia, nitrates, phosphates, and total bacterial counts (see Holmer *et al*., 2008).

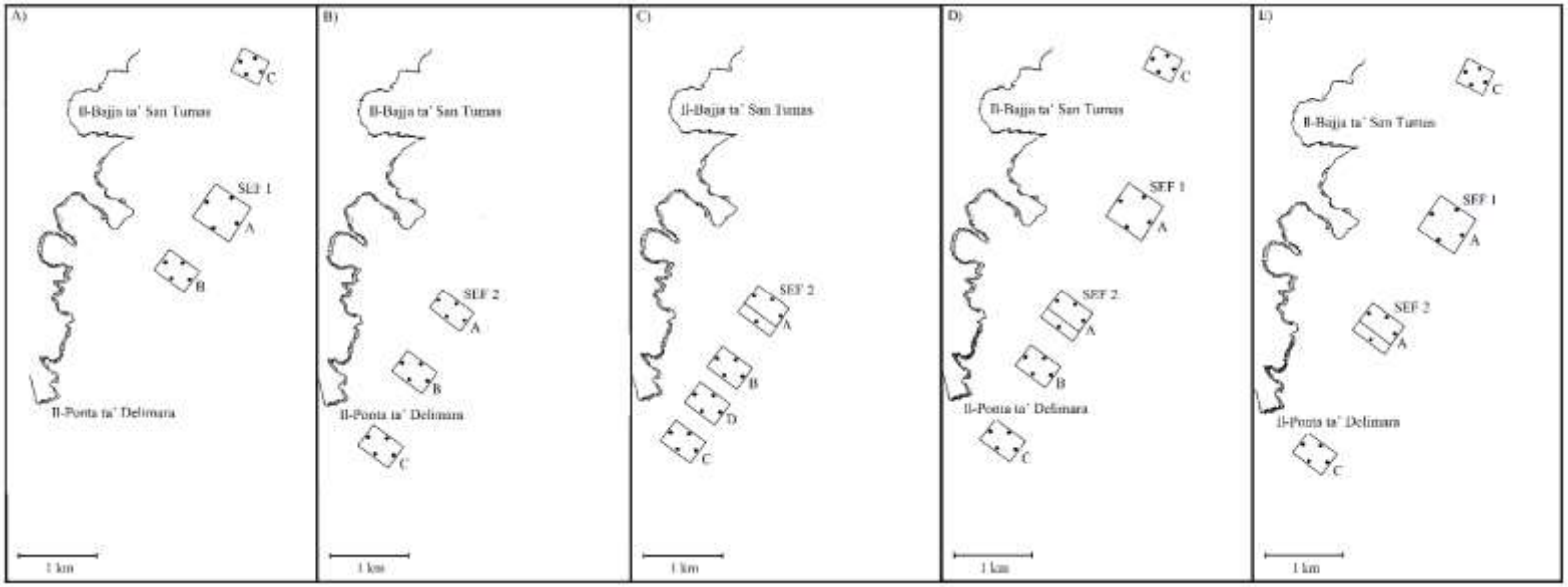


**Figure 1.4** Map of the southeastern study area showing the locations of: (a) Plots A, B, and C at southeastern 'Farm 1' (SEF 1) that were monitored in October '02 to October '05; (b) Plots A, B, and C at southeastern 'Farm 2' (SEF 2) that were monitored in June '01 to June '03; and (c) the extended Plot A, and Plots B, D, and C at the SEF 2 that were monitored in June '04 and '05. The environmental monitoring programme was revised to incorporate common control sites for the two adjacent southeastern tuna farms by eliminating the SEF 2 Plot D in June '06 (d); and the SEF 2 Plot B in June '07 (e).

**Table 1.3** The semi-quantitative index that was developed by Borg and Schemrbi (2005) to quantify and compare the accumulation of uneaten feed-fish on the sediment underneath different tuna cages (see Holmer *et al*., 2008). FF = feed-fish.

| Index Value | Accumulation of uneaten feed-fish on the sediment |
|---|---|
| 0 | 0 FF/$m^2$ of sediment |
| 1 | < 1 FF/$m^2$ of sediment |
| 2 | > 1 FF/$m^2$ of sediment, but not as a continuous layer |
| 3 | > 1 FF/$m^2$ of sediment, as a single continuous layer |
| 4 | > 1 FF/$m^2$ of sediment, as multiple continuous layers |

Unpublished reports of monitoring of benthic habitat undertaken between 2000 and 2008 have indicated occasional significant changes in the abundance and species richness of macrofauna in the vicinity of some of the tuna farms. However, this was mostly limited to the seabed area directly below the tuna pens, and appeared to result from the significantly elevated levels of sediment organic carbon and organic nitrogen content, or/and a significant change in mean grain size of sediments at the same sites and during the same sessions duirng which monitoring of benthic biota has been carried out (see Holmer *et al*., 2008). Where an adverse influence was recorded, this was largely attributed to the accumulation of uneaten feed-fish on the sediment directly below the tuna pens. Some megafauna that are characteristic of bare sand habitat, and which were present on the seabed below the cages before initiation of the tuna penning activities, disappeared and were replaced by dense populations of detrivorous and scavenging macroinvertebrates. The amount of uneaten feed that accumulated on the seabed varied greatly between cages within the same farm, over and above the expected variation between tuna farms. During the fallow period, the remains of uneaten feed-fish formed a layer of decaying organic matter which became admixed with the underlying sediment under the influence of bottom currents, and also probably dispersed to adjacent areas. Concomitantly, a slow benthic recovery was noted, as indicated by the return of some megafauna that were characteristic of the original assemblages present on the seabed prior to deployment of the cages (Borg & Schembri, 2001).

No changes in the biological and physical features of the important benthic habitats and dive sites were detected during the benthic mapping and videographic surveys (see Holmer *et al*., 2008).

The findings from the water quality surveys indicated that, at times, lower oxygen levels, higher turbidity and increased nutrient levels were recorded at the tuna farm sites during the tuna penning season, but such recorded changes in water quality were not statistically significant (see Holmer *et al*., 2008).

### 1.3.4 Environmental monitoring of aquaculture under the EU's WFD

During the past couple of decades, the European Parliament has been stressing the importance of protecting the environment. Of direct relevance to aquaculture activities are the Water Framework Directive (WFD; 2000/60/EC) (European Union [EU], 2000; European Comission [EC], 2003) and the Marine Strategy Framework Directive (MSFD; 2008/56/EC) (EU, 2008), given that the two directives call for community action in water protection and management, and in marine environmental policy, respectively. The determination of the Ecological Quality Status (EQS) of coastal waters under the WFD is based on four biological quality elements; invertebrate fauna, angiosperms, macroalgae and phytoplankton. Ultimately, the WFD calls for classification of coastal water bodies into one of five ecological quality statuses: 'High', 'Good', 'Moderate', 'Poor', or 'Bad'.

As part of the implementation of the WFD in Europe (EC, 2009), several indices that use indicator taxa and which have been derived from the sensitivity/tolerance concept of macroinvertebrate fauna along an organic enrichment gradient (see Section 1.1.3) (e.g. Borja, Franco, & Pérez, 2000; Dauvin & Ruellet, 2007; Simboura & Zentos, 2002), as well as multimetric indices that combine the indicator taxa approach with traditional diversity indices (e.g. Borja *et al*., 2004b; Muxika, Borja, & Bald, 2007), have been developed to measure the EQS of marine waters. These indices have been used in monitoring of aquaculture activities (e.g. Aguado-Giménez *et al*. 2007; Borja *et al*., 2009c; Edgar, Davey, & Shepherd, 2010; Karakassis *et al*., 2013; Katsiaras, Evagelopoulos, Atsalaki, Koutsoubas, & Simboura, 2010; Muxika, Borja, & Bonne,

2005). However, further studies are needed to fill the gaps of knowledge on the performance of these biotic indices compared to traditional data analysis, given the regional variation in the species composition and structure of macroinvertebrate assemblages under different environmental conditions (e.g. Aguado-Giménez *et al*., 2015; Simboura & Argyrou, 2010).

### 1.3.5 Future of environmental monitoring of aquaculture in the Maltese Islands as an EU Member State

The national aquaculture strategy for the Maltese Islands as an EU member state is aimed at sustainable fish farming for the period 2014-2025, so as to meet the requirements of 'Good' Ecological and Environmental Status of coastal waters under the WFD and the MSFD, respectively (Ministry for the Environment, Sustainable Development and Climate Change [MESDC], 2014). The national aquaculture strategy highlights the need for improvement in the environmental monitoring and spatial planning of aquaculture operations (MESDC, 2014). As a result, several operational aquaculture sites in Maltese waters have been proposed as AZAs (Figure 1.2), and the environmental monitoring programs of individual aquaculture operations located near each other, have changed from individual management models to ones that include several farms as a unit (MESDC, 2014). The recommended measures include assessment of the ecological carrying capacities of AZAs, and streamlining of existing environmental monitoring programs (MESDC, 2014). The national aquaculture strategy stresses the need for improved environmental monitoring to be practical and cost effective, and to set Environmental Quality Standards (EnQS) that specify what constitutes adverse infleunce, as well as establishing the Allowed Zone of Effects (AZE) over which such impacts are tolerated (MESDC, 2014).

As part of the implementation of the EU's WFD, the macroinvertebrate indicator taxon index AMBI originally developed in the Basque country (North Spain) (Borja *et al*., 2000) and proposed as an international indicator of aquaculture benthic influence (Draft Standards for Responsible Salmon Aquaculture [DSRSA], 2010), has also been tested in local coastal waters (MEPA, 2013).

## 1.4 Present study

### 1.4.1 Data

Data on attributes of the benthic macrofaunal assemblages and sediment quality (i.e.: mean sediment grain size, and percentage organic carbon and percentage organic nitrogen content of the sediment) have been collected between 2000 and 2009 (Figures 1.3 & 1.4) for the three tuna farms that were sited circa 1 km off the coast of Malta (Figure 1.2), by independent consultants as part of the environmental monitoring programmes described above (Section 1.3.3). The gathered information is considered unique in that it probably comprises the largest and most complete set of data collected in relation to environmental monitoring of tuna penning activities for any region worldwide. Although the results of the various monitoring components have been made available by the independent consultants through reports submitted regularly to the tuna farm operators and the MEPA, the submitted documents only deal with reporting on individual monitoring sessions and do not incorporate detailed statistical analyses of the data or assessments that integrate data from different monitoring sessions. Therefore, a main objective of the present work was to analyse the extensive data set made available to the present author to enable integrated assessment of the influence of tuna farms on benthic habitat in their vicinity. The underwater videographic monitoring data for assessing the gross biological and physical features of the seabed below the tuna pens was not available for consideration in the present work.

### 1.4.2 Aims of the present study

The main aim of the present study was to establish the influence of three offshore tuna farms located circa 1 km off the coast of Malta (Figure 1.2) on the macroinvertebrate assemblages of the soft sediment habitat present in their vicinity. The findings of this research is expected to increase our knowledge and understanding of the environmental influence of ABT ranching on benthic habitat. In turn, such information will help coastal planners and managers to take informed decisions and formulate

effective environmental impact monitoring strategies for aquaculture activities in the Maltese Islands and beyond.

Seven objectives were considered in the present study. The first objective was to assess the influence of a tuna farm located off the northeastern coast on benthic assemblages in its vicinity during its first year of operations, and to test for signs of recovery of the seabed during the fallow period. The NEF; i.e., the largest farm in terms of holding capacity; was selected from the three tuna farms for the present study aspect since lower benthic ecological quality is expected at fish farm sites that have a higher total annual production (Borja, Ranasinghe, & Weisberg, 2009b), and this farm was therefore deemed appropriate for study. Futhermore, six-monthly monitoring data were only available for the NEF. An important aspect of this study component was comparison of data on attributes of the macroinvertebrate assemblages collected before initiation of the activities, with data collected afterwards, which is unique in studies on the influence of ABT farming on the benthos. Such before-after impact data for the NEF was available only for Plots A, B and C (Figure 1.3.a). Since Plot B was no longer considered as a suitable control site in 2001 (see Section 1.3.3; Figure 1.3.b), only data collected from below the pens and from sites located within Plots A and C was included in the experimental design of this study aspect (Chapter 2).

The second objective was to assess the usefulness of the polychaete, mollusc, amphipod and decapod taxocenes, as indicators of environmental change resulting from ABT farming activities. Since different levels of benthic influence are reported in the literature for different ABT farms, an important aspect of this study component was inclusion of a number of tuna farms, reference sites and sampling times in the experimental design to assess the overall influence of tuna penning The selection of after-impact data collected during the years 2003, 2004 and 2005 from the three tuna farms at a site located in the immediate vicinity of the tuna pens and from reference sites located some 1-2 km away (respectively Plots E & C at the NEF; see Figure 1.3.c,d, and Plots B & C at the SEF 1; see Figure 1.4.a, and SEF 2; see Figure 1.4.b), was carried out to reduce the confounding effects of the influence of tuna penning with natural temporal variation, on the bare, soft bottom habitat. The present study

component will help selection of faunal group/s that best signal environmental change resulting from ABT farming activities, thereby decreasing the taxonomic effort needed in environmental impact monitoring studies (Chapter 3).

The third objective was to assess for differences in the level and spatial extent of benthic influence of the three tuna farms that differ in size, stocking density and feed management, as well as in their location. The following null hypothesis was tested: attributes of the polychaete and amphipod assemblages do not differ significantly over time in response to tuna penning activities. An important aspect of this study component was inclusion of multiple spatial scales that ranged from tens of meters to a few kilometers, and which allows determination of the appropriate spatial scale at which potential environmental influence of aquaculture should be investigated. Selection of monitoring data for the present study component was based on the number of years of operation of the tuna farm at the time of sample collection; i.e. it was ensured that this was the same for all farms, to eliminate potential variation that may result from differences in characteristics of the farm and of the receiving environment. A before-after impact-control model was adopted using data collected in: November 2000 and November 2001 at the NEF (Figure 1.3.a, b); October 2002 and October 2003 at the SEF 1 (Figure 1.4.a); and June 2001 and June 2002 at the SEF 2 (Figure 1.4.b) (Chapter 4).

The fourth objective was to test for a potential pattern in attributes of the benthic assemblages with distance from the tuna pens. The spatial pattern in benthic biotic diversity at fish farm sites does not always follow the classical P-R model due to differences in the characteristics of the fish farms, and of the receiving environment. The null hypothesis of no significant difference in attributes of the polychaete and amphipod assemblages over time, with incremental distances of 0 m, 100 m, 1 km and 2 km, from two tuna farms, was tested in the present study component using monitoring data collected at the end of the production period (in autumn) respectively from below the pens, and from sites located in: (i) Plot A and AD, Plot C and Plot E, at the NEF (Figure 1.3.c, d); and (ii) Plot A, Plot B and Plot C, at the SEF 1 (Figure 1.4.a); in the period 2003 to 2005. Grab samples at the SEF 2 were collected in June after the fallow period. The SEF 2 was excluded from this study aspect since

differences in the spatial pattern of tuna penning influence on macroinvertebrate assemblages from the P-R model are expected since recovery of the benthic habitat is expected to take place following cessation of fish farming activities (Fernandes *et al*., 2001) (Chapter 5).

The fifth objective concerned assessment of the suitability of the AMBI, BENTIX, BOPA, BOPA-Fish farming (BOPA-FF) and M-AMBI indices developed under the EU's WFD, for use in the Maltese Islands. An important aspect of this study component was to select data on attributes of the macroinvertebrate assemblages collected from incremental distances of 0 m, 100 m, 1 km, and 2 km from the tuna farms, and which show a well-defined spatial pattern of benthic influence resulting from tuna penning activities, against which the suitability of the WFD biotic indices for use in the Maltese Islands could be tested. As a result, the same benthic monitoring data selected for Chapter 5 was used in this study component. Furthermore, the usefulness of these indices was compared to traditional analyses of attributes used in aquaculture monitoring studies (Chapter 6).

The sixth objective concerned examination of temporal patterns in benthic assemblages in the vicinity of three tuna farms, during a ten year study period. The data used in this specific study component included multiple reference sites and data collected before and after initiation of tuna penning activities. The null hypothesis of no significant difference in BOPA-FF, M-AMBI, and attributes of the polychaete and amphipod assemblages under the influence of tuna penning over time, was tested. Since the environmental monitoring programme at the tuna farms changed several times during the period 2000 to 2009 (Section 1.3.3), different reference sites located some 1 km and 2 km away from the farms had to be selected from time to time, i.e.: (i) Plots B and C in November 2000 and March 2001 (Figure 1.3.a), and Plots C and E in November 2001 to April 2009 (Figure 1.3.b-d), at the NEF; (ii) Plots B and C in October 2002 to 2005 at the SEF 1 (Figure 1.4.a); (iii) Plots B and C in June 2001 to 2005 (Figure 1.4.b, c) at the SEF 2; (iv) and the SEF 1 Plot C and SEF 2 Plot C, in June 2006 to 2009 (Figure 1.4.d, e). Establishing a temporal pattern in biological attributes of benthic assemblages at ABT ranching sites is important given that fish farming may lead to a cumulative type of disturbance and significantly damage

sediment ecological function, rendering fish farming unviable in the long term (Chapter 7).

The seventh and final objective was to integrate the findings and interpretations of the various components of the proposed research with current knowledge of the influence of aquaculture activities and in particular, ABT farming activities, on soft bottom macrofaunal assemblages. The implications of the findings for environmental monitoring and impact mitigation strategies of tuna penning activities on soft bottom habitat were assessed in the Mediterranean, and in a more local context, the Maltese Islands. Evaluating current environmental management practices and challenges for marine aquaculture activities and proposing recommendations for future research, will help coastal managers and researchers identify aspects that require further study for achieving environmentally sustainable tuna penning activities (Chapter 8).

# CHAPTER 2

# INFULENCE OF TUNA PENNING ACTIVITIES ON SOFT BOTTOM MACROBENTHIC ASSEMBLAGES

Part of this chapter has been published as:

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and presented at the Aquaculture Europe 2014 International Conference and Exposition of the European Aquaculture Society, at San Sebastian, Spain, on the 14-17th October 2014.

## 2.1 Introduction

Aquaculture is an important and rapidly expanding food-producing sector (FAO, 2010; International Union for Conservation of Nature [IUCN], 2010) but the activity has been often criticised (Allsopp *et al*., 2008; Esmark & Cripps, 2003) because of its potential adverse influence on the environment, including deterioration of water quality and changes to the biotic assemblages in the vicinity of fish farms (Hargrave *et al*., 1997; GESAMP, 1990; Wu, 1995). A lucrative sector of the aquaculture industry is the ranching of Atlantic Bluefin Tuna (ABT) *Thunnus thynnus thynnus* Linnaeus 1758. ABT has a very high commercial value and constituted 8% of the total global fish exports in 2010 (FAO, 2012), the main producers in the Mediterranean being Italy, Malta and Spain (ICCAT, 2011). A general review of the issues related to the ranching of ABT in the Mediterranean is available in FAO (2005).

The fish are caught in May–July and transferred to offshore floating cages for fattening until October/January (Camilleri, 2017), where they are overfed with fresh fish and molluscs (Aguado *et al*., 2004; Vita & Marin, 2007). The main source of pollution of the seabed at tuna farms are uneaten fish which accumulate below the tuna-pens (Aguado *et al*., 2004; Aguado-Giménez *et al*., 2006; Borg & Schembri, 2005; Vita & Marin, 2007; Vita *et al*., 2004a) and lead to potential adverse effects on the composition and structure of benthic assemblages in the vicinity of a farm (Borg & Schembri, 2005; Vezzulli *et al*., 2008; Vita & Marin, 2007; Vita *et al*., 2004a). Potential adverse effects may be reduced or eliminated when the tuna pens are located well offshore in high energy environments (Aksu *et al*., 2016; Maldonado *et al*., 2005; Moraitis *et al*., 2013). Although ABT is kept in high stocking densities that entail high feed input, the number of stocked tuna varies amongst different farms and even between different cages within the same farm. As a result, one would expect large differences in the level of influence, if present. Furthermore, because of the particular characteristics of the activity, namely use of feed-fish instead of processed feed and the large size of the fish, the potential impacts of tuna penning are expected to differ from those of other intensive fish farming activities, such as salmon, sea bream and sea bass farming.

Studies have assessed the amounts of organic waste generated by ABT farming (Aguado *et al*., 2004; Aguado-Giménez *et al*., 2006; Vita *et al*., 2004a), and the influence of this waste on nutrient levels in the water column and sediment (Aksu *et al*., 2010, 2016; Dal Zotto *et al*., 2016; Marin *et al*., 2007; Matijević *et al*., 2006, 2008; Vezzulli *et al*., 2008; Vita & Marin, 2007), and on water column microbial levels (Kapetanović, Dragun, Vardić Smrzlić, Valić, & Teskeredžić, 2013). Kružić *et al*. (2014) assessed the influence of ABT farming on *Posidonia oceanica* meadows, while Šegvić Bubić *et al*., (2011) evaluated the influence of an ABT farm on the associated wild fish assemblages. Other studies assessed the food-web effects of tuna penning on trophic linkages (Forrestal *et al*., 2012) and the emissions that result from the tuna penning industry (Hospido & Tyedmers, 2005). However, few studies have addressed the influence of ABT ranching on the benthic macroinvertebrate assemblages in the vicinity of tuna pens (Jahani *et al*., 2012; Marin *et al*., 2007; Moraitis *et al*., 2013; Vezzulli *et al*., 2008; Vita & Marin, 2007). In particular, studies comparing attributes of benthic assemblages before initiation of tuna penning to after, are lacking.

The present study aspect was aimed at assessing the influence of a large tuna farm, located off the northeastern coast of the Maltese Islands, on the soft bottom macroinvertebrate assemblages present in its vicinity. The farming practice included a fallow period during winter of each year when the pens did not hold any tuna. Samples of soft sediment for biological and physico-chemical studies were collected in autumn and spring before initiation of the tuna penning activities and after during the same seasons. The following null hypothesis was tested: tuna penning activities do not have an influence on (a) sediment physico-chemical attributes and (b) number of taxa, abundance of selected macroinvertebrate taxa, and assemblage composition of the macroinvertebrates associated with the soft sediment habitat in the vicinity of tuna pens.

## 2.2 Material and methods

### 2.2.1 Study area and sampling

The tuna farm studied was located 1 km off the northeastern coast of the Maltese Islands (Figure 2.1), where the seabed consisted of soft sediment and the water depth was between 45 m and 50 m.

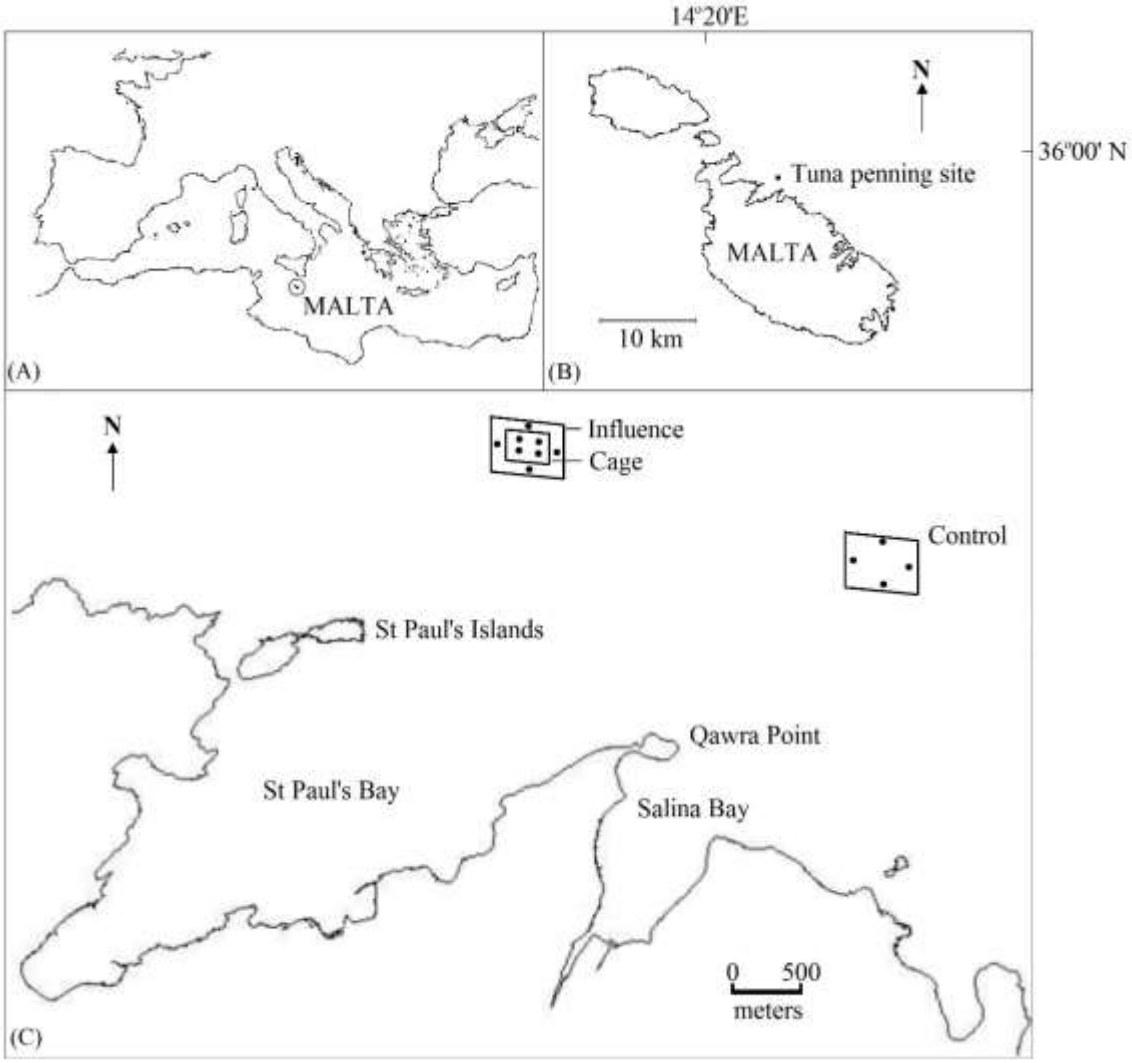


**Figure 2.1** Map showing: (a) the Maltese Islands at the center of the Mediterranean; (b) the location of the study area off the northeastern coast of the Maltese Islands; and (c) locations of the Cage, Influence and Control areas used in the present study.

The farm had a total annual capacity of around 2500 t and utilised cages of 25 m height and 50 m diameter. The sampling design incorporated three sampling areas at incremental distances from the tuna pens, all of which had a similar bottom type: (i) 'Cage' area, i.e. the seabed area directly beneath the tuna cages; (ii) 'Influence' area, some 200 m from the cages; and (iii) 'Control' area, some 1.5 km from the cages. Four replicate sampling sites were allotted to each area, such that a total of 12 samples were collected on each sampling occasion. The latitude/longitude coordinates and depth of the sampling sites are shown in Table 2.1. Sampling was carried out in November (hence autumn) 2000 and in March (hence spring) 2001 before initiation of any tuna penning activities, and one year later in November 2001 and in April 2002 following commencement of the tuna penning activities. The cages did not hold any tuna during the fallow period in winter.

**Table 2.1** Latitude/longitude coordinates and depth of the Influence and Control sites shown in Figure 2.1. The Cage area is centered on N35º 58.75'/E14º 25.16', and samples were also collected from the seabed area directly below the cages.

| Area | Site | Latitude/longitude | Depth (m) |
|---|---|---|---|
| Influence | a | N35º 58.77/E14º 25.18 | 48 |
| | b | N35º 58.63/E14º 25.31 | 48 |
| | c | N35º 58.56/E14º 25.18 | 48 |
| | d | N35º 58.70/E14º 25.03 | 48 |
| Control | a | N35º 58.32/E14º 26.72 | 50 |
| | b | N35º 58.18/E14º 26.85 | 50 |
| | c | N35º 58.12/E14º 26.72 | 48 |
| | d | N35º 58.26/E14º 26.59 | 50 |

Samples were collected using a 0.1 $m^2$ van Veen grab. Three replicate grab samples for benthic macrofaunal studies and one grab sample for sediment studies were collected from each of the twelve sampling sites in November 2000, March 2001, November 2001 and April 2002 (Figure 2.1). As the exact place where the cages would be located was not yet known at the time when the 'Before' samples were collected, sampling in November 2000 and March 2001 was made in the general area of influence of the farm, rather than from the specific locations indicated in Figure 2.1 in the area where the cages are found. Samples for sediment analyses were collected in March 2001, November 2001 and April 2002, but not in November 2000.

In the laboratory, samples for faunal studies were sorted for macroinvertebrates after washing on a 0.5 mm mesh. Macroinvertebrates were identified to family level and enumerated to obtain estimates of number of taxa and abundance per grab sample. For sediment physico-chemical studies, sub-samples for the determination of percent organic nitrogen content (PONC), percent organic carbon content (POCC) and weight/weight percent feed-fish bone content (FFBC) were frozen at - 20°C for later analysis, while another sub-sample was oven-dried for determination of mean sediment grain size (MSGS). Analysis of the sediment to determine the FFBC was carried out by sorting fish bones from the sediment using forceps under a dissecting microscope. PONC in the sediment was determined using the Kjeldhal method (see Holme & McIntyre, 1984), while POCC in the sediment was determined using acid digestion (see Walkley & Black, 1934). MSGS was determined according to Buchanan (1984).

### 2.2.2 Data analyses

Separate three-factor univariate permutational analysis of variance (PERMANOVA) (Anderson, 2001; McArdle & Anderson, 2001) was carried out on the number of taxa and abundance of selected indicator taxa Paraonidae (Polychaeta), Phoxocephalidae (Amphipoda), Apseudidae (Tanaidacea), and Arcidae (Bivalvia) using a model with two orthogonal factors 'Before/After' (BA; 2 levels, before and after, fixed) and 'Area' (Ar; 3 levels, Cage, Influence and Control, fixed), and the factor 'Site' (Si; 4 levels, a–d, random) nested within 'BA x Ar', using data collected in (i) November 2000 and November 2001, and (ii) March 2001 and April 2002. The four indicator taxa at family level were selected on the basis of being the four most abundant macroinvertebrate families in the data collected before the tuna penning activities were initiated. Separate two-factor univariate PERMANOVA, based on a model with the two orthogonal factors 'Before/After' and 'Area' where levels of 'Site' were treated as replicates, was carried out using sediment data for MSGS, POCC and PONC, collected in (i) March 2001 and November 2001, and (ii) March 2001 and April 2002. Missing data on physico-chemical sediment attributes for November 2000 was replaced with that collected in March 2001, with the assumption that natural seasonal factors did not influence sediment attributes.

To test for differences in the macroinvertebrate assemblage composition, three-factor multivariate PERMANOVA (Anderson, 2001; McArdle & Anderson, 2001) was run (with the level of significance [α] set at 0.05) using the Bray Curtis similarity matrix calculated from the family abundance data which were fourth-root transformed to downweigh very abundant taxa (Clarke & Warwick, 2001). A permutational multivariate dispersion test (PERMDISP) (Anderson, 2004, 2006) (with α set at 0.05) was then used to test for significant differences in dispersion of samples using the sample distance to the centroid of each of the factors. In both PERMANOVA and PERMDISP tests, a total of 9999 unrestricted permutations of raw data (Anderson, 2005) were used and *a posteriori* pair-wise comparisons were carried out to investigate differences among groups (with α set at 0.05) for the highest order of interaction. Principal coordinate analysis (PCO) (Anderson, 2003) was run and the results plotted. To test for differences in sediment attributes, similar multivariate analyses were run, using the two-factor model, on a D1 Euclidean similarity matrix calculated from environmental data that was normalised to homogenize the different units (Clarke & Warwick, 2001). The three most important taxa contributing to dissimilarities between assemblages after initiation of tuna penning were identified using the similarity percentages of species contributions (SIMPER) method (Clarke & Warwick, 2001), and *a posteriori* three-factor ANOVA was run (with α set at 0.05) on the abundance of the taxa that contributed most to the dissimilarities. To determine which sediment physico-chemical attribute, or combination of attributes, best explained the potential differences in the macroinvertebrate assemblages after initiation of tuna penning, biota and/or environment matching (BIOENV) analysis (Clarke & Gorley, 2006) was carried out, using the Spearman rank correlation method and D1 Euclidean similarity measure. The multivariate analyses were carried out using PRIMER 6 v.6.1 (Clarke & Gorley, 2006) and PERMANOVA+ v.1.0 add-on package (Anderson, Gorley & Clarke, 2008).

## 2.3 Results

### 2.3.1 Univariate data analyses

#### 2.3.1(i) Macroinvertebrate assemblages

A total of 18,292 macroinvertebrates, from 128 macroinvertebrate families were recorded. In general, there was an increase in the mean abundance of Paraonidae, Apseudidae and Arcidae, and a decrease in the abundance of Phoxocephalidae in autumn after initiation of tuna penning (Figure 2.2). Paraonidae and Apseudidae peaked in abundance some 200 m away from the cages in the same period. Conversely, the abundance of Paraonidae, Phoxocephalidae and Apseudidae after initiation of tuna penning were lower in the following spring, while that of Arcidae was higher (Figure 2.2).

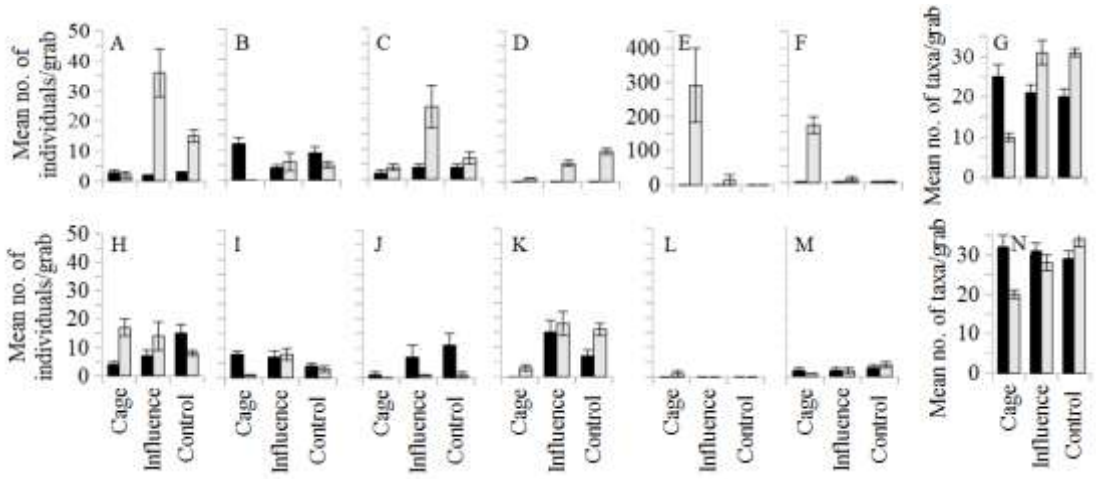


**Figure 2.2** Mean number of individuals (±SE) per grab of: (a, h) Paraonidae, (b, i) Phoxocephalidae, (c, j) Apseudidae, (d, k) Arcidae, (e, l) Mactridae and (f, m) Capitellidae, and (g, n) the mean number of taxa (±SE) per grab, recorded before initiation of tuna penning activities (black bars), and after (grey bars) in (a–g) November 2000 and November 2001, and (h–n) March 2001 and April 2002, from the Cage, Influence and Control areas.

Univariate PERMANOVA indicated significant difference in the abundance of Paraonidae ($p < 0.001$), Phoxocephalidae ($p < 0.05$), Apseudidae ($p < 0.05$) and Arcidae ($p < 0.001$) for the spatio-temporal interaction 'BA x Ar' in autumn (November 2000 and November 2001) (Table 2.2). *A posteriori* pair-wise tests indicated significant decrease in the abundances of Paraonidae ($p < 0.05$) and

Phoxocephalidae ($p < 0.001$) at the Cage area; and significant increase in the abundances of Paraonidae, Apseudidae and Arcidae at the Influence area ($p < 0.01$), and in the abundances of Paraonidae ($p < 0.05$) and Arcidae ($p < 0.01$) at the Control area, in autumn after initiation of tuna penning (Table 2.2). PERMANOVA also indicated significant small-scale spatial variation ('Si(BA x Ar)') in the abundance of Phoxocephalidae ($p < 0.01$) and Arcidae ($p < 0.05$) in the same period (Table 2.2).

PERMANOVA indicated significant difference in the abundance of Paraonidae ($p < 0.05$) and Phoxocephalidae ($p < 0.01$) for 'BA x Ar' in spring (March 2001 and April 2002) (Table 2.2). *A posteriori* pair-wise tests indicated that the abundance of Paraonidae increased significantly ($p < 0.05$) at the Cage area in spring after the tuna penning, while the abundance of Phoxocephalidae at the Cage area decreased significantly ($p < 0.01$) in the same period (Table 2.2).

PERMANOVA indicated significant temporal variation ('BA') in the abundance of Apseudidae ($p < 0.01$) and Arcidae ($p < 0.05$) in spring. The abundance of Arcidae also showed significant spatial variation ('Ar', $p < 0.001$) in the same period (Table 2.2). Pair-wise tests showed that the abundance of Apseudidae decreased significantly ($p < 0.05$) over all three areas after the tuna penning; while the abundance of Arcidae increased significantly ($p < 0.05$) over all three areas after initiation of tuna penning, and was significantly higher ($p < 0.001$) at the Influence and Control areas than at the Cage area in spring (Table 2.2). PERMANOVA also indicated significant small-scale spatial variation ('Si(BA x Ar)', $p < 0.01$; Table 2.2) in the abundance of Paraonidae in the same period.

In general, the mean total number of taxa recorded per grab in autumn after tuna penning was lower at the Cage area compared to that recorded at the Influence and Control areas, while values of this same attribute were higher in the following spring at the Cage area (Figure 2.3).

PERMANOVA indicated significant spatio-temporal variation ('BA x Ar') in the total number of taxa in autumn (November 2000 and November 2001) ($p < 0.001$) and in spring (March 2001 and April 2002) ($p < 0.05$) (Table 2.2). *A posteriori* pair-wise

**Table 2.2** Results of the three-factor univariate PERMANOVA and *a posteriori* pair-wise tests for number of individuals (NI) of the indicator taxa Paraonidae (1), Phoxocephalidae (2), Apseudidae (3) and Arcidae (4), number of individuals of Mactridae (Mac) and Capitellidae (Cap), and total number of families (NFa). Level of significance set at 0.05. Df = Degrees of freedom, ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001

| | | | November 2000 and November 2001 | | | | | | March 2001 and April 2002 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | | df | NI 1 | NI 2 | NI 3 | NI 4 | Mac | Cap | NI 1 | NI 2 | NI 3 | NI 4 | Mac | Cap |
| Before/After = BA | | 1 | *** | * | * | *** | ns | *** | ns | * | ** | * | ns | ns |
| Area = Ar | | 2 | *** | ns | * | *** | ns | *** | ns | ** | ns | *** | ns | * |
| BA x Ar | | 2 | *** | * | * | *** | ns | *** | * | ** | ns | ns | ns | ns |
| Site = Si(BA x Ar) | | 18 | ns | ** | ns | * | *** | ** | * | ns | ns | ns | * | ns |
| Residual | | 48 | | | | | | | | | | | | |
| Total | | 71 | | | | | | | | | | | | |
| *Pair-wise tests -: 'BA' and 'Ar'* | | | | | | | | | | | | | | |
| Before vs After | | | - | - | - | - | - | - | - | - | > * | < * | - | - |
| Cage vs Influence | | | - | - | - | - | - | - | - | - | - | < *** | - | ns |
| Cage vs Control | | | - | - | - | - | - | - | - | - | - | < *** | - | < * |
| Influence vs Control | | | - | - | - | - | - | - | - | - | - | ns | - | ns |
| *Pair-wise tests-: 'BA x Ar'* | | | | | | | | | | | | | | |
| Before vs After | Cage | | > * | > *** | ns | ns | - | < ** | < * | > *** | - | - | - | - |
| | Influence | | < *** | ns | < * | < ** | - | ns | ns | ns | - | - | - | - |
| | Control | | < *** | ns | ns | < ** | - | ns | ns | ns | - | - | - | - |
| Cage vs Influence | Before | | ns | > ** | ns | ns | - | ns | ns | ns | - | - | - | - |
| | After | | < ** | ns | < * | < ** | - | > ** | ns | < * | - | - | - | - |
| Cage vs Control | Before | | ns | ns | ns | ns | - | ns | < ** | > * | - | - | - | - |
| | After | | < ** | > ** | ns | < ** | - | > ** | ns | ns | - | - | - | - |
| Influence vs Control | Before | | ns | ns | ns | ns | - | ns | ns | ns | - | - | - | - |
| | After | | ns | ns | ns | ns | - | ns | ns | ns | - | - | - | - |

tests showed that the number of taxa decreased significantly ($p < 0.05$) at the Cage area, and increased significantly at the Influence ($p < 0.01$) and Control ($p < 0.001$) areas, in autumn after initiation of tuna penning; and was significantly lower ($p < 0.05$) at the Cage area compared to the Influence and Control areas in spring after the tuna penning (Table 2.2). PERMANOVA also detected significant small-scale spatial variation ('Si(BA x Ar)', $p < 0.01$; Table 2.2) in the total number of taxa in the same period

**2.3.1(ii) Sediment physico-chemical attributes**

Fish bones from uneaten feed-fish that had accumulated and decomposed on the seabed during the tuna penning season, were only recorded in samples collected from below the cages, where they were notably more abundant in the autumn after initiation of tuna penning than in the following spring (Figure 2.3). There was a particularly high accumulation (some 6% w/w) of FFBC in the sediment below one of the four cages ('Cage b') in autumn after tuna penning, where POCC (0.946%) and PONC (0.126%) were particularly high, and MSGS was particularly low (0.96 mm), in the same period. In general, POCC levels varied greatly between sediment samples collected from below the cages, while high PONC levels were recorded below the cages compared to values at the Influence and Control areas both in autumn after initiation of tuna penning and the following spring (Figure 2.3). MSGS was higher in sediment samples collected in autumn after the tuna penning from below the cages, but lower in the following spring (Figure 2.3).

Univariate PERMANOVA indicated significant difference in MSGS ($p < 0.05$) and POCC ($p < 0.01$) for the spatio-temporal interaction 'BA x Ar' in autumn (March 2001 and November 2001) (Table 2.3). *A posteriori* pair-wise tests indicated significant increase in MSGS ($p < 0.05$) and POCC ($p < 0.01$) at the Cage area, and in POCC at the Influence ($p < 0.001$) area, in autumn after initiation of tuna penning (Table 2.3). PERMANOVA indicated significant temporal variation ('BA', $p < 0.01$; Table 2.3) in PONC in autumn. Pair-wise tests showed that PONC increased significantly ($p < 0.05$) overall at all three areas in autumn after the tuna penning activities (Table 2.2).

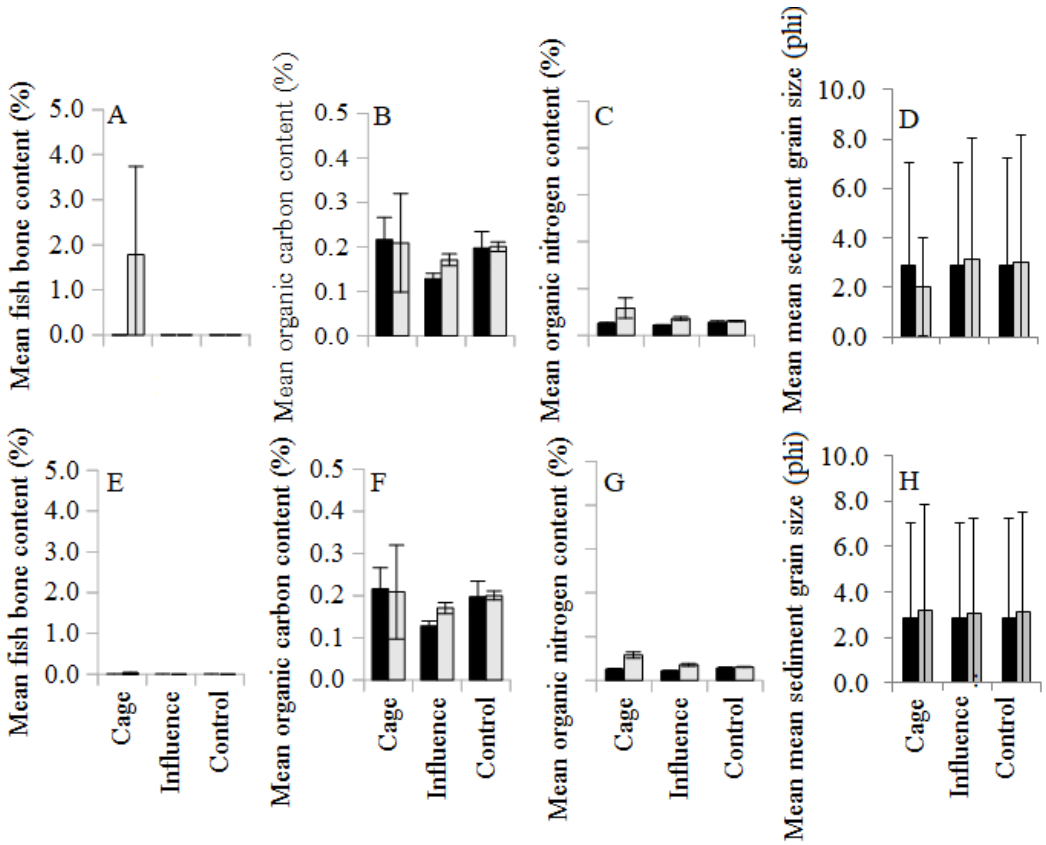


**Figure 2.3** Mean values (±SE) of feed-fish bone content per grab recorded in (a) November 2001 and (e) April 2002 below the cages; and mean values (±SE) per grab of (b, f) percent organic carbon content, (c, g) percent organic nitrogen content, and (d, h) mean sediment grain size (phi) recorded before initiation of tuna penning activities (black bars), and after (grey bars) in (a–d) November 2000 and November 2001, and (e–h) November 2000 and April 2002, from the Cage, Influence and Control areas.

PERMANOVA indicated significant spatio-temporal variation ($p < 0.01$) in PONC for 'BA x Ar' in spring (March 2001 and April 2002), and *a posteriori* pair-wise tests showed that PONC increased significantly ($p < 0.01$) in samples collected from the Cage and Influence areas in spring after tuna penning (Table 2.3). PERMANOVA also indicated significant temporal and spatial variation in MSGS ('BA' and 'Ar', $p < 0.01$; Table 2.3). The pair-wise tests showed significantly decreased ($p < 0.01$) MSGS in samples collected from all three areas in spring after initiation of tuna penning, and significantly lower ($p < 0.01$) MSGS at the Cage area compared to Influence and Control areas in spring (Table 2.3).

**Table 2.3** Results of the two-factor univariate PERMANOVA and *a posteriori* pair-wise tests for mean sediment grain size (phi) (MSGS), and percent organic carbon content (POCC) and organic nitrogen content (PONC) in sediment. Level of significance set at 0.05. Df = Degrees of freedom, ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001

| | | | March 2001 and November 2001 | | | March 2001 and April 2002 | |
|---|---|---|---|---|---|---|---|
| Source of Variation | df | MSGS | POCC | PONC | MSGS | POCC | PONC |
| Before/After = BA | 1 | ns | *** | ** | ** | ns | *** |
| Area = Ar | 2 | ns | *** | ns | ** | ns | ** |
| BA x Ar | 2 | * | ** | ns | ns | ns | ** |
| Residual | 18 | | | | | | |
| Total | 23 | | | | | | |
| *Pair-wise tests -: 'BA' and 'Ar'* | | | | | | | |
| Before vs After | | - | - | < * | > ** | - | - |
| Cage vs Influence | | - | - | - | < ** | - | - |
| Cage vs Control | | - | - | - | < ** | - | - |
| Influence vs Control | | - | - | - | ns | - | - |
| *Pair-wise tests-: 'BA x Ar'* | | | | | | | |
| Before vs After | Cage | < * | < ** | - | - | - | < ** |
| | Influence | ns | < *** | - | - | - | < ** |
| | Control | ns | ns | - | - | - | ns |
| Cage vs Influence | Before | < * | ns | - | - | - | ns |
| | After | ns | > * | - | - | - | > * |
| Cage vs Control | Before | < ** | ns | - | - | - | ns |
| | After | ns | > ** | - | - | - | > ** |
| Influence vs Control | Before | ns | ns | - | - | - | ns |
| | After | ns | > * | - | - | - | Ns |

### 2.3.1(iii) Relationship between sediment attributes and macroinvertebrates

BIOENV indicated that POCC best explained the observed pattern in the abundance of Mactridae ($p < 0.01$) and Capitellidae, and in the number of families ($p < 0.001$) in autumn after initiation of tuna penning (Table 2.4). BIOENV also showed that in the following spring, PONC best explained ($p < 0.01$) the pattern in the number of families after initiation of tuna penning (Table 2.4). The Spearman rank correlation coefficient for the total number of families was larger in autumn after initiation of tuna penning ($\rho = 0.780$) than in the following spring ($\rho = 0.566$). There was no significant correlation with the MSGS, POCC or PONC of the sediment, for the abundance of the selected indicator taxa in autumn and in spring, and for the abundance of Capitellidae and Mactridae in spring following the tuna penning (Table 2.4).

**Table 2.4** Results of BEST analysis showing the best explanatory variable (Best Exp Var) or combination thereof correlated with the number of individuals of the indicator taxa Paraonidae (1), Phoxocephalidae (2), Apseudidae (3) and Arcidae (4), number of individuals of Mactridae (Mac) and Capitellidae (Cap), and total number of families (NFa) in autumn (November 2001) and in spring (April 2002) after the tuna penning activities. Level of significance set at 0.05. A Euclidean similarity matrix was used for biotic data. ρ-value = Spearman's rank correlation coefficient, p(PERM) = Permutational p-value, POCC = percent organic carbon content, PONC = percent organic nitrogen content, MSGS = mean sediment grain size (phi), ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$

| | November 2001 | | | April 2002 | | |
|---|---|---|---|---|---|---|
| | ρ-value | p(PERM) | Best Exp Var | ρ-value | p(PERM) | Best Exp Var |
| NI 1 | 0.22 | ns | POCC | 0.18 | ns | POCC |
| NI 2 | 0.22 | ns | MSGS | 0.22 | ns | MSGS, POCC |
| NI 3 | 0.07 | ns | MSGS | 0.34 | ns | MSGS |
| NI 4 | 0.36 | ns | MSGS, POCC | 0.30 | ns | MSGS, POCC, PONC |
| Mac | 0.69 | ** | POCC | 0.37 | ns | MSGS, POCC, PONC |
| Cap | 0.91 | *** | POCC | 0.14 | ns | PONC |
| NFa | 0.78 | *** | POCC | 0.57 | ** | PONC |

### 2.3.2 Multivariate data analyses

#### 2.3.2(i) Macroinvertebrate assemblages

PCO ordination of family abundance data indicated clear separation between groups of samples, however, the first and second PCO axes together explained only half (48.5%) of the total variation in family abundance (Figure 2.4). Samples collected from below the cages in autumn after initiation of tuna penning activities were distinctly separated from all other samples by the second PCO axis (Figure 2.4). Samples collected in autumn before tuna penning commenced from the three areas were placed in a group that was separated by the first PCO axis from the group of samples collected in spring before tuna penning commenced from the three areas. The other distinct sample groups were: the group collected in autumn after initiation of tuna penning and in the following spring from the Influence and Control areas; and the group of samples collected in spring after tuna penning from the Cage area (Figure 2.4).

PERMANOVA indicated that the square root estimate of residual variation as a component of variation (33.103) was approximately as large as that for 'BA' (32.002) and 'BA x Ar' (29.672) in autumn (Table 2.5). PERMANOVA also indicated that the

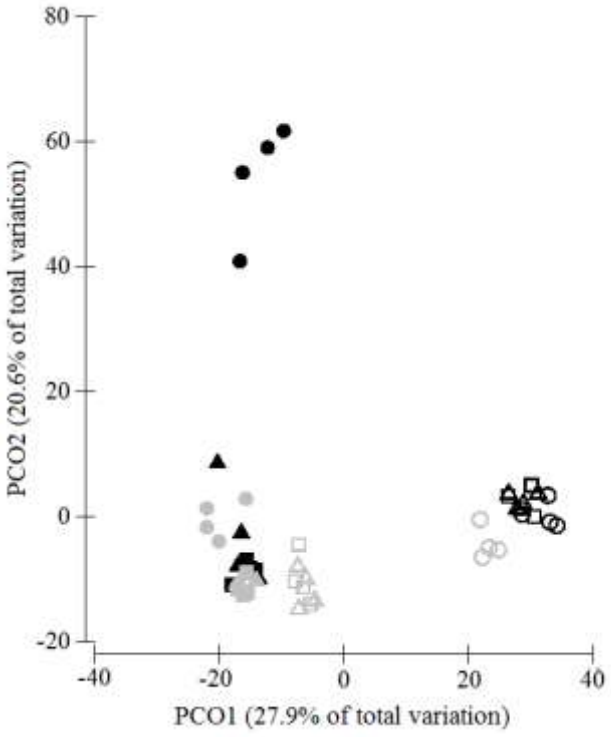


**Figure 2.4** PCO plot calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data recorded from: the Cage (circle), Influence (triangle), and Control (square) areas, before initiation of tuna penning activities (unshaded) in November 2000 (black) and March 2001 (grey), and afterwards (shaded) in November 2001 (black) and April 2002 (grey).

square root estimate of residual variation as a component of variation was much larger (30.484) than the next important ones ('BA', 23.182; 'BA x Ar', 20.442) in spring (Table 2.5). Both PERMANOVA and PERMDISP tests indicated significant differences for 'BA x Ar' in autumn (November 2000 and November 2001) ($p_{PERMANOVA} < 0.001$; $p_{PERMDISP} < 0.01$), and in spring (March 2001 and April 2002) ($p_{PERMANOVA\ BA\ x\ Ar} < 0.001$; $p_{PERMDISP\ BA\ x\ Ar} < 0.01$) (Table 2.5). *A posteriori* pair-wise tests showed that assemblage composition differed significantly at Cage, Influence and Control areas in autumn ($p < 0.001$) and in spring ($p_{PERMANOVA} < 0.001$; $p_{PERMDISP\ Cage} < 0.01$, $_{Control} < 0.05$) following initiation of tuna penning, and differed significantly between the Cage area, and the Influence ($p_{PERMANOVA} < 0.001$; $p_{PERMDISP} < 0.05$) and Control ($p < 0.001$) areas in the same period. Assemblage composition also differed significantly ($p < 0.05$) between Influence and Control areas in spring after the tuna penning (Table 2.5). Both PERMANOVA and PERMDISP tests also indicated significant small-scale spatial variation in autumn ($p_{PERMANOVA} < 0.001$; $p_{PERMDISP} < 0.05$) and in spring ($p_{PERMANOVA} < 0.01$; $p_{PERMDISP} < 0.001$) (Table 2.5).

**Table 2.5** Results of three-factor PERMANOVA and three-factor PERMDISP calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data, with *a posteriori* pair-wise comparisons. Variables included in this analysis are fourth-root transformed family abundance. Level of significance set at 0.05. Df = Degrees of freedom, Sqr Rt Var = Square Root Estimate of Component of Variation, p(PERM) = Permutational p-value, p(MC) = Monte Carlo p-value, p(Tables) = p-value from tables, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| November 2000 and November 2001 | | | | |
|---|---|---|---|---|
| | | | PERMANOVA | PERMDISP |
| Source of Variation | df | Sqr Rt Var | p(PERM) | p(PERM) |
| Before/After = BA | 1 | 32.002 | *** | ns |
| Area = Ar | 2 | 19.211 | *** | ** |
| BA x Ar | 2 | 29.672 | *** | ** |
| Site = Si(BA x Ar) | 18 | 12.784 | *** | * |
| Reisudal | 48 | 33.103 | | |
| Total | 71 | | | |
| *Pair-wise tests -: 'BA x Ar' using p(MC) and p(Tables)* | | | | |
| Before vs After | Cage | | *** | ns |
| | Influence | | *** | ns |
| | Control | | *** | *** |
| Cage vs Influence | Before | | ** | * |
| | After | | *** | ns |
| Cage vs Control | Before | | ** | ** |
| | After | | *** | ns |
| Influence vs Control | Before | | ns | ns |
| | After | | ns | ns |
| March 2001 and April 2002 | | | | |
| | | | PERMANOVA | PERMDISP |
| Source of Variation | df | Sqr Rt Var | p(PERM) | p(PERM) |
| Before/After = BA | 1 | 23.182 | *** | ** |
| Area = Ar | 2 | 17.16 | *** | *** |
| BA x Ar | 2 | 20.442 | *** | ** |
| Site = Si(BA x Ar) | 18 | 8.9913 | ** | *** |
| Residual | 48 | 30.484 | | |
| Total | 71 | | | |
| *Pair-wise tests -: 'BA x Ar' using p(MC) and p(Tables)* | | | | |
| Before vs After | Cage | | *** | ** |
| | Influence | | *** | ns |
| | Control | | *** | * |
| Cage vs Influence | Before | | *** | ** |
| | After | | * | ns |
| Cage vs Control | Before | | *** | ns |
| | After | | *** | ** |
| Influence vs Control | Before | | ns | ns |
| | After | | * | * |

SIMPER showed that no single taxon made a large contributory influence to the dissimilarities between samples collected from the three areas in autumn after initiation of tuna penning; the same held true for samples collected in the following spring (Table 2.6). SIMPER indicated a high dissimilarity for samples collected in autumn after tuna penning between the Cage area, and the Influence and Control areas. The taxa that contributed to this dissimilarity were Mactridae (Bivalvia) and Capitellidae (Polychaeta), both of which were more abundant at the Cage area than at the Influence and Control areas. In the following spring, SIMPER indicated that Mactridae and Capitellidae were no longer important contributing taxa to the dissimilarity between the Cage area, and the Influence and Control areas. For samples collected from the Influence and Control areas, SIMPER indicated that the average dissimilarity between the autumn after tuna penning and the spring after tuna penning was much lower than for samples collected from the Cage area (Table 2.6).

*A posteriori* PERMANOVA indicated a significant difference ($p < 0.01$) in the abundance of Capitellidae for 'BA x Ar' in autumn (November 2000 and November 2001) (Table 2.1). PERMANOVA also indicated a significant difference in the abundance of Capitellidae ($p < 0.01$) and Mactridae ($p < 0.001$) for 'Si(BA x Ar)' in the same period. Pair-wise tests showed that the abundance of Capitellidae at the Cage area increased significantly ($p < 0.01$) in autumn following initiation of tuna penning activities, and was significantly higher ($p < 0.01$) than at Influence and Control areas in the same period (Table 2.1).

*A posteriori* PERMANOVA indicated a significant spatial variation ('Ar', $p < 0.05$) in the abundance of Capitellidae in spring (March '01 and April '02), and in the abundance of Mactridae for 'Si(BA x Ar)' in the same period (Table 2.1). Pair-wise tests indicated a significantly low ($p < 0.05$) abundance of Capitellidae at the Cage area compared to the Control area in spring (Table 2.4).

### 2.3.2(ii) Sediment physico-chemical attributes

PCO ordination of sediment data explained almost all the total variation (92.6%) in sediment quality and showed that separation between groups of samples on the PCO axes was small (Figure 2.5). The first PCO axis explained half of the total variation

**Table 2.6** SIMPER results calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data, showing the top three contributing taxa (in terms of number of individuals) to the dissimilarity in macroinvertebrate assemblages recorded between Cage, Influence and Control areas in November 2001 and April 2002, and between November 2001 and April 2002 at each of the three areas. Avg Diss (%) = Average Dissimilarity, Av Abund = Average Abundance, Contrib (%) = Contribution (%)

| Level | Groups | Avg Diss (%) | Family | Av Abund a | Av Abund b | Contrib (%) |
|---|---|---|---|---|---|---|
| November 2001 | Cage (a), Influence (b) | 67.65 | Mactridae | 3.64 | 0.88 | 5.11 |
| | | | Capitellidae | 3.58 | 1.52 | 3.84 |
| | | | Clathurellidae | 0.00 | 1.67 | 3.09 |
| | Cage (a), Control (b) | 71.71 | Mactridae | 3.64 | 0.19 | 6.33 |
| | | | Capitellidae | 3.58 | 1.26 | 4.29 |
| | | | Clathurellidae | 0.00 | 1.44 | 2.66 |
| | Influence (a), Control (b) | 33.95 | Photidae | 0.19 | 1.19 | 2.61 |
| | | | Trochidae | 0.87 | 0.00 | 2.28 |
| | | | Mactridae | 0.88 | 0.19 | 2.27 |
| April 2002 | Cage (a), Influence (b) | 42.44 | Maldanidae | 0.42 | 1.37 | 2.68 |
| | | | Hesionidae | 0.00 | 0.93 | 2.61 |
| | | | Phoxocephalidae | 0.75 | 1.66 | 2.56 |
| | Cage (a), Control (b) | 44.58 | Hesionidae | 0.00 | 1.23 | 3.04 |
| | | | Maldanidae | 0.42 | 1.43 | 2.52 |
| | | | Ophiuridae | 0.27 | 1.30 | 2.50 |
| | Influence (a), Control (b) | 31.50 | Nephtyidae | 0.19 | 1.15 | 2.91 |
| | | | Ophiuridae | 0.42 | 1.30 | 2.63 |
| | | | Aoridae | 0.19 | 0.95 | 2.24 |
| Cage | November 2001 (a), April 2002 (b) | 82.72 | Mactridae | 3.58 | 0.31 | 9.98 |
| | | | Capitellidae | 3.54 | 0.52 | 9.62 |
| | | | Clathurellidae | 0.00 | 1.52 | 4.66 |
| Influence | | 49.41 | Apseudidae | 2.03 | 0.45 | 4.33 |
| | | | Maldanidae | 0.35 | 1.17 | 2.58 |
| | | | Arcidae | 1.31 | 1.94 | 2.17 |
| Control | | 43.51 | Ophiuridae | 0.00 | 1.20 | 3.43 |
| | | | Apseudidae | 1.36 | 0.42 | 3.11 |
| | | | Maldanidae | 0.36 | 1.41 | 3.05 |

in sediment quality (55.8%), and accounted for separation of the group of samples collected in autumn after initiation of tuna penning at the Cage area from the groups of samples collected in the same period from the Influence and Control areas (Figure 2.5). The second PCO axis explained most of the remaining total variation (36.8%) and separated the samples collected in spring after initiation of tuna penning from the Cage area from the group of samples collected in the same period from the Influence and Control areas (Figure 2.5).

PERMANOVA ($p < 0.05$) and PERMDISP ($p < 0.001$) tests detected a significant difference ($p < 0.01$) in physico-chemical attributes of the sediment for 'BA x Ar' in

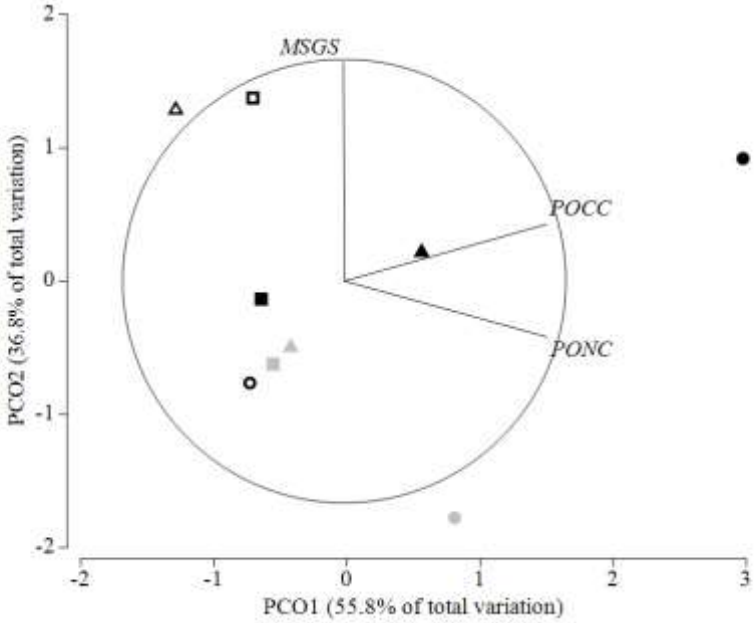


**Figure 2.5** PCO plot calculated from a Euclidean similarity matrix of normalised sediment physico-chemical data recorded from: the Cage (circle), Influence (triangle), and Control (square) areas, before initiation of tuna penning activities (unshaded) in March 2001 (grey), and afterwards (shaded) in November 2001 (black) and April 2002 (grey).The plot also shows overlaid vectors of sediment 'MSGS' mean sediment grain size (phi), 'POCC' percent organic carbon content and 'PONC' percent organic nitrogen content having a Pearson correlation > 0.5.

autumn (March 2001 and November 2001), and for 'BA' ($p < 0.001$) and 'Ar' ($p < 0.01$) in spring (March 2001 and April 2002) (Table 2.7). For these, PERMANOVA gave very small (c. 1) values for the square root estimates of each interaction term and factor as components of variation, while the residual variation in sediment quality unexplained by the multivariate model was small (Table 2.7).

Pair-wise comparisons of PERMANOVA indicated a significant difference in sediment quality between the Cage area, and the Influence ($p < 0.05$) and Control ($p < 0.01$) areas, before tuna penning commenced (Table 2.7). Sediment quality differed significantly ($p < 0.05$) at the Cage and Influence areas between samples collected before tuna penning commenced and those collected after initiation of tuna penning in autumn. Sediment quality differed significantly ($p < 0.05$) between the Cage area and the Control area in autumn after tuna penning (Table 2.7). Sediment quality differed significantly ($p < 0.05$) at the Cage and Influence areas between samples collected before tuna penning commenced and those collected in spring after initiation of tuna

**Table 2.7** Results of the two-factor multivariate PERMANOVA and PERMDISP tests calculated from a Euclidean similarity matrix of normalised sediment physico-chemical data, with *a posteriori* pair-wise comparisons for the significant second order interaction term. The variables are: normalised mean sediment grain size (phi), and percent organic carbon content and percent organic nitrogen content in the sediment. Level of significance set at 0.05. Df = Degrees of freedom, Sqr Rt Var = Square Root Estimate of Component of Variation, p(PERM) = Permutational p-value, p(MC) = Monte Carlo p-values, p(Tables) = p-values from tables, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| November 2000 and November 2001 | | | | |
|---|---|---|---|---|
| | | | PERMANOVA | PERMDISP |
| Source of Variation | | df | Sqr Rt Var | p(PERM) | p(PERM) |
| Before/After = BA | | 1 | 0.92623 | *** | ns |
| Area = Ar | | 2 | 0.70649 | ** | ns |
| BA x Ar | | 2 | 1.1864 | ** | ** |
| Residual | | 18 | 1.3098 | | |
| Total | | 23 | | | |
| *Pair-wise tests -: 'BA x Ar' using p(MC) and p(Tables)* | | | | | |
| Before vs After | Cage | | | * | * |
| | Influence | | | * | ns |
| | Control | | | ns | ns |
| Cage vs Influence | Before | | | * | ns |
| | After | | | ns | ns |
| Cage vs Control | Before | | | ** | ns |
| | After | | | * | ns |
| Influence vs Control | Before | | | ns | ns |
| | After | | | ns | ns |
| March 2001 and April 2002 | | | | | |
| | | | PERMANOVA | PERMDISP |
| Source of Variation | | df | Sqr Rt Var | p(PERM) | p(PERM) |
| Before/After = BA | | 1 | 1.0602 | *** | ns |
| Area = Ar | | 2 | 0.90925 | ** | ns |
| BA x Ar | | 2 | 0.6718 | ns | ns |
| Residual | | 18 | 1.2967 | | |
| Total | | 23 | | | |
| *Pair-wise tests -: 'BA x Ar' using p(MC) and p(Tables)* | | | | | |
| Before vs After | Cage | | | * | - |
| | Influence | | | * | - |
| | Control | | | ns | - |
| Cage vs Influence | Before | | | * | - |
| | After | | | * | - |
| Cage vs Control | Before | | | ns | - |
| | After | | | * | - |
| Influence vs Control | Before | | | ns | - |
| | After | | | ns | - |

penning. Sediment quality differed significantly ($p < 0.05$) between the Cage area and the Influence and Control areas in spring after tuna penning (Table 2.7).

Pair-wise comparisons using the PERMDISP test indicated no significant difference in sediment quality between the Cage, Influence and Control areas before tuna penning commenced (Table 2.7). Sediment quality differed significantly ($p < 0.05$) between samples collected before tuna penning and ones collected in autumn after tuna penning at the Cage area (Table 2.7).

### 2.3.2(iii) Relationship between sediment attributes and macroinvertebrates

BIOENV indicated that POCC best explained ($p < 0.01$) the observed multivariate pattern in autumn after initiation of tuna penning. BIOENV also indicated that in the following spring, PONC best explained ($p < 0.001$) the multivariate pattern. The Spearman rank correlation coefficient was larger in autumn after initiation of tuna penning ($\rho = 0.803$) than in the following spring ($\rho = 0.643$).

## 2.4 Discussion

Significant changes in sediment quality and in the total number of macrofaunal taxa, abundance of selected macroinvertebrate taxa, and the macroinvertebrate assemblage structure were recorded in the vicinity of the tuna pens following commencement of tuna penning; these changes appear to have resulted from the large amount of feed-fish that accumulates on the seabed in the vicinity of the tuna pens (Holmer *et al*., 2008). Some of this material is dispersed to adjacent areas, resulting in changes to the soft bottom habitat at distances of some 200 m away from the cages.

The spatial extent of the influence of tuna penning activities on benthic assemblages recorded in the present study component is larger than that reported for Mediterranean sea bream and sea bass farms (e.g. Di Marco *et al*., 2017; Karakassis *et al*., 2000; Karakassis, Tsapakis, Smith, & Rumohr, 2002; Tomassetti *et al*., 2016), and for a tuna farm located off the southeastern coast of Spain (Marin *et al*., 2007), but similar to that for another tuna farm located off the southeastern coast of Spain (Vita & Marin, 2007). The high biomass of fish held at tuna farms, the tendency for an overfeeding regime aimed at reaching elevated lipid contents, and the poor food conversion ratio (Aguado-Giménez *et al*., 2006), all contribute to higher organic loading of the seabed under the

pens. Tuna faeces dissolves more quickly while settling through the water column compared to the faeces of other smaller farmed fish species; hence the rate of deposition of particulate waste at tuna farms is lower than that at farms holding other fish species (Vita *et al*., 2004a). On the other hand, this implies that tuna waste may be dispersed more readily by currents, such that the area with deposited particulate organic matter may extend well beyond the farm lease areas (Fernandes *et al*., 2007b).

The observed significant changes in sediment quality and attributes of the macroinvertebrate assemblages were conspicuous during autumn, towards the end of the tuna penning season. At the Cage area, where large amounts of uneaten feed-fish had accumulated on the seabed, sediment organic carbon and mean grain size increased significantly, while the total number of taxa decreased significantly and the abundance of Capitellidae increased significantly. At the Influence area some 200 m from the cages, sediment organic carbon also increased significantly. On the other hand, no significant changes in attributes of sediment quality were recorded at the Control area some 1.5 km from the cages. However, percent organic nitrogen content in the sediment increased significantly at all three areas, while the total number of taxa increased significantly at both Influence and Control areas.

Capitellid polychaetes are opportunistic (Borja *et al*., 2000) and notoriously abundant in the vicinity of fish farms (e.g. Karakassis *et al*., 2000; Vita & Marin, 2007). These and other opportunistic taxa characteristically dominate the macroinvertebrate benthic assemblages present in the vicinity of a source of organic enrichment (Pearson & Rosenberg, 1978) and therefore serve as good indicators of organic pollution. Uneaten feed-fish and tuna waste that accumulate and decompose on the sediment below tuna pens result in enhanced microbial activity that leads to high redox sediment conditions (Giles, 2008). Such adverse conditions are tolerated only by specialised opportunistic species and result in reduced benthic macrofaunal diversity (Giles, 2008). Studies similar to the present work have shown a minimum in diversity and a maximum in dominance for macroinvertebrates just below the cages during the tuna penning season (Jahani *et al*., 2012; Marin *et al*., 2007; Vita & Marin, 2007), where Capitellidae flourished (Vita & Marin, 2007).

In the present study aspect, Capitellidae and Mactridae (suspension-feeding bivalves), which were not recorded in the study area before tuna penning commenced, were abundant below the cages in autumn, towards the end of the farming season. On the other hand, the abundance of Paraonidae (deposit-feeding polychaetes) and Phoxocephalidae (deposit-feeding amphipods) decreased significantly below the cages. During the same period, the abundance of Apseudidae (surface deposit-feeding tanaids) increased significantly at the Influence area some 200 m from the tuna cages, as did the abundance of Paraonidae and Arcidae (suspension-feeding bivalves). Significant differences in the abundance of the indicator taxa were also detected at the Control area some 1.5 km from the cages, where the abundance of Paraonidae and Arcidae increased significantly. In spring, after a few months had elapsed following harvesting of the tuna and cessation of ranching activities, the abundance of Paraonidae increased significantly below the cages, while the abundance of Arcidae (suspension-feeding bivalves) increased significantly at the Influence and Control areas, but was significantly low at the Cage area. The abundance of Phoxocephalidae decreased significantly below the cages, while the abundance of Apseudidae decreased significantly over all three areas.

At tuna farms, the output of organic waste is highest during the production period that lasts around 6 months from June to December, and is followed by a fallow period (January to May). Cessation of organic inputs to the marine environment during the latter period may be expected to contribute to an overall reduced influence on the environment (e.g. Macleod *et al.*, 2006, 2007). However, tuna farms have been claimed to produce seven times the annual gross waste output of gilthead sea bream or sea bass farms, even though these have a longer production period of some 16 or 18 months and contribute to continuous organic loading of the marine environment (Aguado-Giménez *et al.*, 2006). It would seem that following harvesting of the tuna in December, most of the uneaten feed-fish that would have accumulated below the tuna pens during the farming period decomposes, and only fish bones and other organic material persist on the seabed. Storms and currents help disperse and mix this organic material; as a result, there is some recovery and the state of sediments and of the macrobenthic assemblages revert to those that characterised the benthic habitat prior to initiation of penning activity. However, the sediment organic nitrogen remained significantly high below the cages in spring after the tuna penning, where the total

number of taxa was significantly low in the same period. In soft sediment habitats having high levels of total organic carbon, the diversity of associated benthic macrofauna is low but the converse is true at low levels of the attribute (Hyland *et al*., 2005). In the oligotrophic Mediterranean Sea, relatively low levels of sediment organic matter have been recorded below tuna cages (Vita & Marin, 2007). However, this renders the benthic ecosystem more sensitive to organic enrichment.

A decrease in organic waste accumulated in the sediment below the cages during the tuna penning season and signs of recovery of benthic habitat during the fallow period have been reported for other Mediterranean tuna farms (Marin *et al*., 2007; Vita & Marin, 2007). On the other hand, Vita and Marin (2007) concluded that the six month long fallow period was not enough for complete recovery of benthic assemblages below tuna cages at the farm they investigated. Nonetheless, the seasonality of tuna penning together with the often offshore locations of tuna-penning installations allow for some benthic recovery that mitigates potentially adverse environmental influence (Aksu *et al*., 2010, 2016; Moraitis *et al*., 2013; Vezzulli *et al*., 2008; Vita & Marin, 2007). Vezzulli *et al*. (2008) provided evidence to support the importance of using modern offshore farming technology to minimise organic loading associated with tuna penning in the Mediterranean Sea. When located in deep waters characterised by high energy conditions, as was the case for the tuna farm considered in the present study component, tuna penning operations in the Mediterranean can have much less adverse influence on the water column, sediment quality and macrobenthic fauna (Aksu *et al*., 2016; Moraitis *et al*., 2013; Vezzulli *et al*., 2008). Aguado *et al*. (2004) argued for the need for an artificial formulated diet for tuna penning and for a controlled feeding regime to mitigate the level of any potential adverse influence. Scavengers attracted to the uneaten feed that falls to the bottom in the vicinity of fish farms also play an important role in the removal of accumulated waste on the seabed, and in recycling organic matter and regulating benthic community structure (Vita *et al*., 2004b). Some 80% of the particulate organic waste deposited in the vicinity of sea bass and sea bream farms in the Mediterranean may be eaten by wild fish before settling (Vita *et al*., 2004b). At tuna farms in South Australia, wild fish contribute to removal of accumulated organic waste on the seabed (Svane & Barnett, 2008), and likewise benthic scavengers in the Mediterranean (Vizzini & Mazzola, 2012). However, Šegvić Bubić *et al*. (2011) noted that the feed-fish fed to penned tuna are not as rapidly

consumed as the pelletized feed used in sea bass and sea bream farms in the Eastern Adriatic Sea. Vizzini and Mazzola (2012) showed that the dilution and dispersion of organic wastes by currents, as well as consumption by scavengers, prevented accumulation of organic matter in the sediment below the tuna cages. Fernandes *et al*. (2007a) estimated that some 3% of feed-fish at tuna farms in South Australia is uneaten and becomes available to scavengers, but noted that most of the organic nitrogen came from tuna faeces, which was the major source of accumulated organic waste in the sediment.

In the present study component, the results of the PCO analysis indicated that, in autumn, the macroinvertebrate assemblages present below one of the four cages differed from those present below the other cages. Sediment quality differed between the different cage sites; the quantity of bones originating from feed-fish, organic carbon content and organic nitrogen content were higher, and mean sediment grain size was lower below this particular cage compared to the other cages. Significant difference in the abundance of taxa which appeared to flourish below the cages, namely Mactridae and Capitellidae, was also recorded for sampling site. These observations clearly demonstrate that feed management differs between cages within the same farm, over and above the expected variation in the feeding regime between different tuna farms.

As far as we are aware, the present study is the first to incorporate comparison of data on benthic assemblages present in the vicinity of a tuna farm collected before initiation of tuna penning with that collected after such activities commenced; this allowed proper assessment of changes to sediment quality and attributes of the soft sediment macrofaunal assemblages present in the immediate vicinity of the farm and at two other areas located at different distances away. Despite being found at an offshore location characterised by deep waters and a high energy environment that aids dispersion and dilution of organic waste originating from the farm, the tuna penning activities resulted in significant changes to sediment physico-chemical attributes and to the macroinvertebrate assemblages of soft bottom habitats located in the immediate vicinity of the fish cages up to a distance of some 200 m away. However, it appears that the magnitude and spatial extent of the influence would also depend on the feed

management regime adopted at tuna farms; hence this aspect should be given high importance and there should be measures to mitigate overfeeding.

# CHAPTER 3

# ASSESSMENT OF BENTHIC BIOLOGICAL INDICATORS FOR EVALUATING THE ENVIRONMENTAL INFLUENCE OF TUNA PENNING

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## 3.1 Introduction

There have been growing concerns about the potential adverse environmental effects of the farming of Atlantic bluefin tuna (ABT), *Thunnus thynnus thynnus* Linnaeus 1758, in the Mediterranean, given the rapid expansion of this activity. A general overview of global Bluefin tuna farming and sustainability concerns is available in Metian, Pouil, Boustany, and Troell (2014), while issues related to the environmental effects of tuna penning in the Mediterranean are reviewed in Food and Agriculture Organization of the United Nations (FAO) (2005). The ranched tuna are overfed with whole bait-fish to achieve high lipid contents (FAO, 2004). Uneaten feed-fish and fish faeces are the main source of pollution of the seabed in tuna penning (Aguado *et al*., 2004; Aguado-Giménez *et al*., 2006; Borg & Schembri, 2005; Mangion, Borg, Thompson, & Schembri, 2014; Vita & Marin, 2007; Vita *et al*., 2004a) and may lead to potential adverse effects on the diversity and structure of benthic assemblages in their vicinity (Borg & Schembri, 2005; Mangion *et al*., 2014; Vita & Marin, 2007; Vita *et al*., 2004a).

Several studies on the potential environmental influence of tuna penning have been carried out in the Mediterranean (e.g. Aguado-Giménez *et al*., 2006; Aksu *et al*., 2010, 2016; Dal Zotto *et al*., 2016; Kružić *et al*., 2014; Matijević *et al*., 2006, 2008; Vita *et al*., 2004a) including ones that have addressed the effects of ABT farming on the macrobenthic assemblages in the vicinity of fish cages at single tuna farms (Jahani *et al*., 2012; Mangion *et al*., 2014; Marin *et al*., 2007; Moraitis *et al*., 2013; Vezzulli *et al*., 2008; Vita & Marin, 2007). Since different levels of environmental influence are reported for different tuna farms (e.g. Jahani *et al*., 2012; Moraitis *et al*., 2013), it would be useful to adopt a design that includes multiple tuna farms and reference areas in studies of the influence of tuna penning on benthic habitats, as this would help to better understand the influence of this activity on the marine environment.

While studies on the influence of ABT farming on benthic habitats have used a variety of biological and physico-chemical attributes as indicators, there is no overall agreement on which indicator best signals environmental change, possibly as a result of insufficient research on this aspect. The polychaete/amphipod (BOPA-Fish farming) ratio (Aguado-Giménez *et al*., 2015) is a benthic biotic index developed for

the European Water Framework Directive (WFD, 2000/60/EC) to classify water bodies into 'High', 'Good', 'Moderate', 'Poor' or 'Bad' Ecological Quality Status (EQS) classes (Dauvin & Ruellet, 2007; Gomez-Gesteira & Dauvin, 2000). Although polychaetes (e.g. Aguado-Giménez *et al*., 2015; Martinez-Garcia *et al*., 2013; Tomassetti & Porrello, 2005) and amphipods (e.g. Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011), as well as molluscs (e.g. Charalampos & Drosos, 2008), have been used to assess the effect of Mediterranean sea bream /sea bass farming on benthic habitats, the performance of these taxa as indicators of the effects of tuna penning on macroinvertebrate assemblages has not been fully explored. Furthermore, studies on the influence of tuna penning on benthic habitats that are based on a design that incorporates more than one tuna farm, which would render a more robust assessment, are lacking.

The present study was aimed at (i) assessing the usefulness of attributes of benthic macroinvertebrate assemblages, namely abundance of indicator taxa, total number of taxa, Shannon-Wiener diversity index, polychaete/amphipod ratio (Aguado-Giménez *et al*., 2015) and composition of the polychaete, mollusc, amphipod and decapod taxocenes, as indicators of potential change resulting from the influence of tuna penning on benthic habitats at different spatial scales, and (ii) using these indicators to establish an overall effect of tuna penning activities on soft bottom habitat over time using data collected from three large tuna farms in the central Mediterranean during a three year period.

## 3.2 Material and methods

### 3.2.1 Study sites and sampling

The three tuna farms considered in the present study were located 1 km off the northeastern to southeastern coast of the Maltese Islands (Figure 3.1) where the seabed consisted of soft sediment, and the water depth ranged between 42–53 m. The northernmost farm (NEF) had eight tuna cages with a maximum total annual capacity of around 2500 t, while the other two farms were smaller and each had a maximum total annual capacity of around 1500 t; one farm (southeastern 'Farm 1'; [SEF 1]) had three cages and the other (southeastern 'Farm 2' [SEF 2]) had four cages (see Figure

3.1). The three farms utilized cages that had a diameter of some 50 m and their base lied at a water depth of around 25 m. The designated areas occupied by the tuna farms were: 350 m x 500 m (NEF), 550 m x 550 m (SEF 1), and 300 m x 500 m (SEF 2). The tuna were stocked at a density of circa 2 to 4 kg $m^{-3}$ and were fed the equivalent of 3-4 % of the fish biomass a day, in two feeding sessions (tuna farm managers, personal communication, January 14, 2015). Bait fish were used as feed; namely mackerel, sardines, squid and prawn; with a food conversion ratio (based on the wet weight of the feed) of 10-15:1 (tuna farm managers, personal communication, January 14, 2015).

The sampling design incorporated three sampling plots which supported the same benthic habitat type at a similar water depth: (i) the 'Impact' plot, i.e. the seabed area occupied by the footprint of the tuna pens; (ii) the 'Control 1' plot, located circa 1 km away from the cages; and (iii) the 'Control 2' plot, located circa 2 km away from the cages (Figure 3.1). Three sampling sites were allotted to each plot, as the smallest of the farms had three cages. This sampling design was replicated at each of the three farms, such that it included a total of 27 sampling sites. The latitude/longitude coordinates and depth of the sampling sites are shown in Table 3.1.

Fish farming activities were initiated during summer in 2001 at the NEF and SEF 2, and in 2003 at the SEF 1. Sampling of the soft sediment assemblages was carried out as part of an environmental monitoring program, as required by the local environmental and planning authority, in November 2003, 2004 and 2005 at the NEF, in October 2003, 2004 and 2005 at the SEF 1, and in June 2003, 2004 and 2005 at the SEF 2, following initiation of tuna penning activities. Samples were collected using a 0.1 $m^2$ van Veen grab. Three replicate grab samples for study of benthic macrofauna and one grab sample for study of sediment physico-chemical attributes were collected at each of the 27 sampling sites. The collected samples were sieved using a 0.5 mm mesh on board the vessel and the material retained by the sieve was preserved in 10 % seawater formalin.

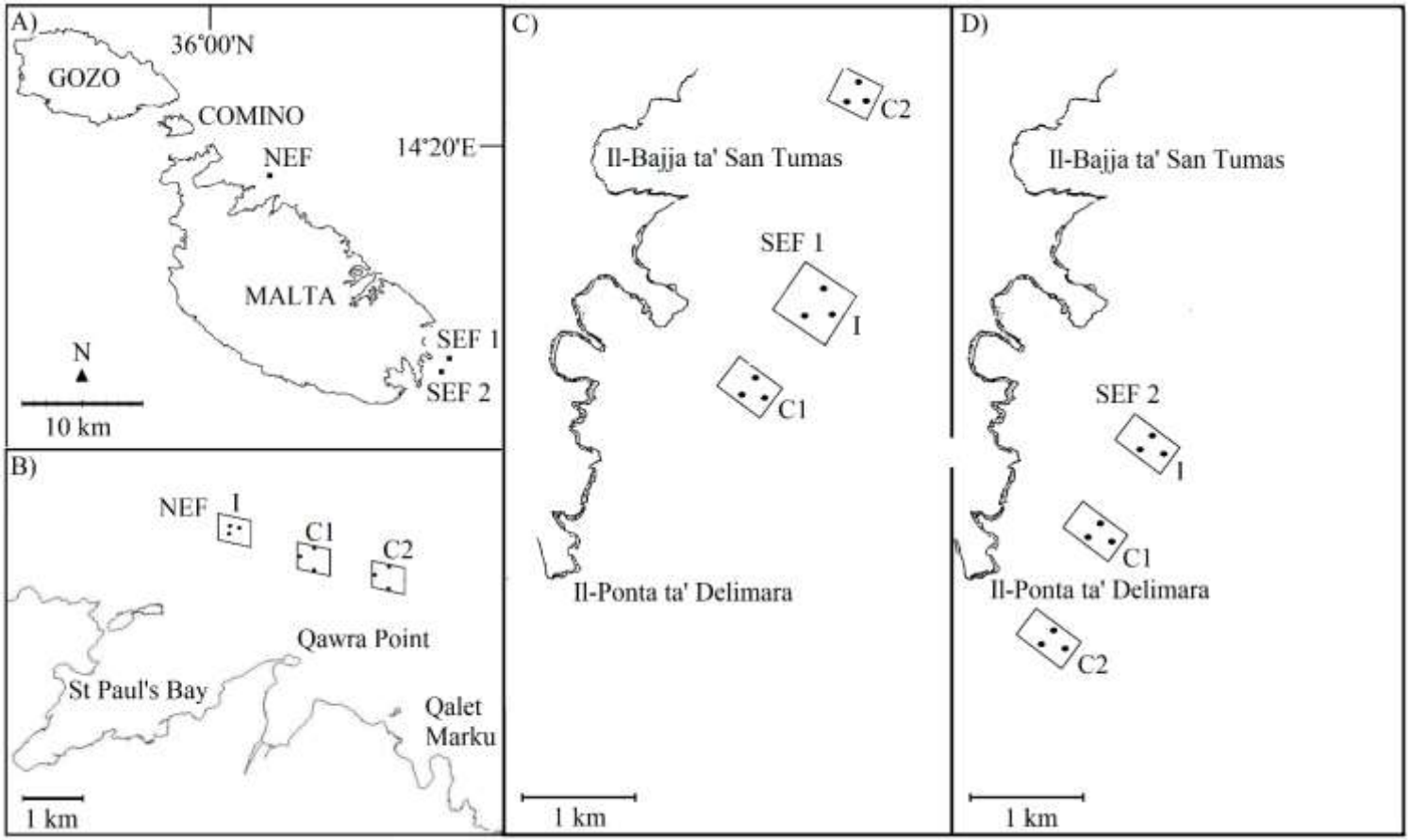


**Figure 3.1** Map of the Maltese Islands showing: (a) the locations of the three tuna farms; (b) the northeastern farm (NEF); (c) southeastern 'Farm 1' (SEF 1); and (d) southeastern 'Farm 2' (SEF 2); from where samples for sediment quality and benthic macrofaunal studies were collected. I = impacted plot (= farm location), C1 = 'Control 1' plot, C2 = 'Control 2' plot

**Table 3.1** Latitude/longitude coordinates and depth of the 18 control sites shown in Figure 3.1. The impacted plot at the northeastern farm (NEF) was centered on: N35º 58.66'/E14º 25.16'; at southeastern 'Farm 1' (SEF 1) on N35º 50.17'/E14º 35.11'; and at southeastern 'Farm 2' (SEF 2) on N35º 49.85'/E14º 34.67'; while samples were also collected from the seabed area directly below the cages.

| | | NEF | |
|---|---|---|---|
| Plot | Site | Latitude/Longitude | Depth (m) |
| 'Control 1' | S1 | N35º 58.32/E14º 26.72 | 50 |
| | S2 | N35º 58.18/E14º 26.85 | 50 |
| | S3 | N35º 58.12/E14º 26.72 | 48 |
| 'Control 2' | S1 | N35º 58.51/E14º 26.33 | 52 |
| | S2 | N35º 58.38/E14º 26.47 | 52 |
| | S3 | N35º 58.32/E14º 26.33 | 51 |
| | | SEF 1 | |
| Plot | Site | Latitude/Longitude | Depth (m) |
| 'Control 1' | S1 | N35º 50.18/E14º 34.79 | 51 |
| | S2 | N35º 50.10/E14º 34.85 | 51 |
| | S3 | N35º 50.11/E14º 34.70 | 46 |
| 'Control 2' | S1 | N35º 51.58/E14º 35.42 | 47 |
| | S2 | N35º 51.50/E14º 35.48 | 47 |
| | S3 | N35º 51.49/E14º 35.37 | 45 |
| | | SEF 2 | |
| Plot | Site | Latitude/Longitude | Depth (m) |
| 'Control 1' | S1 | N35º 49.55/E14º 34.41 | 47 |
| | S2 | N35º 49.35/E14º 34.48 | 47 |
| | S3 | N35º 49.36/E14º 34.28 | 46 |
| 'Control 2' | S1 | N35º 49.02/E14º 34.15 | 48 |
| | S2 | N35º 48.83/E14º 34.22 | 49 |
| | S3 | N35º 48.87/E14º 34.00 | 48 |

In the laboratory, the samples were washed on a 0.5 mm mesh to remove the formalin saline and the retained fauna was sorted. Polychaete, mollusc, amphipod and decapod taxa were identified to family level (see Karakassis & Hatziyanni, 2000; Olsgard & Somerfield, 2000) and counted to obtain estimates of number of families and number of individuals per grab sample. For sediment physico-chemical studies, sub-samples for the determination of percent organic carbon content (POCC), percent organic nitrogen content (PONC) and w/w feed-fish bone content (FFBC) were frozen at -20°C for later analysis, while another sub-sample was oven dried for granulometric analysis. Analysis of the sediment to determine % FFBC was carried out for samples collected from below the fish cages by micro-sorting of the sediment. Analysis of the POCC was carried out by acid digestion (see Walkley & Black, 1934) and of PONC by the Kjeldhal method (see Holme & McIntyre, 1984); while measurement of mean

sediment grain size (MSGS) was made according to the method described by Buchanan (1984) (see Holme & McIntyre, 1984).

### 3.2.2 Data analyses

Data on the polychaete, mollusc, amphipod and decapod taxocenes were analysed as follows; macroinvertebrate indicator taxa at family level were selected as the three families in each taxocene with the highest abundance, contributing most to the difference in assemblage composition between the impacted and control plots using the similarity percentages of species contributions (SIMPER) method (Clarke & Warwick, 2001) (see below): Capitellidae, Maldanidae, and Sabellidae (polychaetes), Cerithiidae, Carditidae and Solemyidae (molluscs), Corophiidae, Urothoidae and Lysianassidae (amphipods), and Paguridae, Galatheidae, and Processidae (decapods). Four-factor permutational univariate analysis of variance (PERMANOVA) (Anderson, 2001) was used (with level of significance [α] set at 0.05) on a Euclidean similarity matrix to test the hypothesis of no significant differences in the abundance of selected indicator taxa, number of taxa and Shannon-Wiener diversity of taxa per faunal group associated with the soft sediment habitat, and the polychaete/amphipod (BOPA-Fish farming) index as defined by Aguado-Giménez *et al*. (2015), under the influence of tuna penning activities over time, using a model with four factors: 'Location' (Lo; 3 levels, NEF, SEF 1 and SEF 2, random), 'Plot' (Pl; 3 levels, with a fixed component, Impact, and two random components, 'Control 1' and 'Control 2'), 'Time' (Ti; 3 levels, 2003, 2004 and 2005, random), and 'Site' (Si; 3 levels, S1, S2 and S3, random) nested within the 'Lo x Pl x Ti' interaction. Separate three-factor univariate PERMANOVA was used (with α set at 0.05) on a Euclidean similarity matrix to test the hypothesis of no differences in the MSGS, POCC and PONC of the sediment, using a similar experimental design, with levels of 'Si' treated as replicates.

An asymmetrical design was adopted for the factor 'Pl' as there was only one impacted plot for every two control plots (Underwood, 1992, 1994). The asymmetrical PERMANOVAs were constructed by combining sum of squares values from three separate PERMANOVAs as described by Glasby (1997), to provide for the partitioning of the factor 'Pl' into two components: the contrast test between the impacted plot and the average of the two control plots ('Impact-vs-Control') ('Im-vs-

Co'), and the variability between the two control plots at each farming location ('Co(Lo)'). The numerator/s and denominator/s used to calculate the F ratio for the individual terms in the three-factor and four-factor asymmetrical PERMANOVAs are given respectively in Tables 3.1 and 3.3.

The main PERMANOVA term of interest that assesses for an overall influence of tuna penning activities on benthic habitat over time is the 'Im-vs-Co x Ti' interaction, while the 'Lo x Im-vs-Co x Ti' interaction indicates variability in the influence of tuna penning activities between farming locations over time. The 'Co(Lo) x Ti' interaction indicates spatial variation (at the scale of 1 km) between control areas over time at each farming location, while the 'Si(Lo x Im-vs-Co x Ti)' and 'Si(Co(Lo) x Ti)' terms indicated temporal variability at the smallest spatial scale (of a few meters).

To test the hypothesis of no difference in the family abundance of the polychaete, mollusc, amphipod and decapod assemblages under the influence of tuna penning activities over time, four-factor permutational multivariate ANOVA (PERMANOVA) (Anderson, 2001; McArdle & Anderson, 2001) was used (with α set at 0.05) on a Bray Curtis similarity matrix calculated from the family abundance data which were fourth-root transformed to downweigh very abundant taxa (Clarke & Warwick, 2001). A permutational multivariate dispersion test (PERMDISP) (Anderson, 2004, 2006) was then used to identify differences (with α set at 0.05) in within-group dispersion using the sample distance to the centroid of each of the factors. In PERMANOVA and PERMDISP analyses, a total of 9999 unrestricted permutations of raw data (Anderson, 2005) were used. Principal Coordinate Analysis (PCO) (Anderson, 2003) was run on a Bray Curtis similarity matrix calculated from fourth-root transformed family abundance data which was averaged at the level of 'Pl x Ti' to plot similarity in macroinvertebrate assemblages between impacted and control groups over time. To test for differences in sediment physico-chemical variables, similar multivariate analyses were run, using the three-factor model, on a D1 Euclidean similarity matrix calculated from environmental data that was normalised to homogenize the different units (Clarke & Warwick, 2001). The three most important taxa contributing to dissimilarity in assemblages between impacted and control groups were identified using SIMPER (Clarke & Warwick, 2001) and an *a posteriori* four-factor univariate PERMANOVA was then run (with α set at 0.05) on the abundance of the taxa that

contributed most notably to the dissimilarities. To determine which sediment physico-chemical variable, or combination of variables, best explained the potential differences in the macroinvertebrate assemblages at the impacted and control plots, the BEST routine of the biota and/or environment matching (BIOENV) analysis (Clarke & Gorley, 2006) was carried out, using the Spearman rank correlation method and D1 Euclidean similarity measure. All the analyses were implemented using PRIMER v.7.0.11 (PRIMER software; Clarke & Gorley, 2006) and the PERMANOVA+ v.1.0 add-on package (Anderson *et al*., 2008).

## 3.3 Results

### 3.3.1 Univariate data analyses

#### 3.3.1(i) Sediment physico-chemical attributes

Values of mean percent FFBC of the sediment recorded below tuna cages at the NEF and SEF 1 were high compared to those recorded at the SEF 2, and decreased overall during the study period (from 0.80 $\pm$ 0.20 % to 0. 34 $\pm$ 0.24 %, and from 1.59 $\pm$ 1.53 % to 0.55 $\pm$ 0.24 %, respectively), while those recorded at the SEF 2 increased (from 0.00 $\pm$ 0.00 % to 0.56 $\pm$ 0.38 %). The general trend in MSGS, POCC and PONC of the sediment over time was similar at impacted and control plots (Figure 3.2) with no significant differences for the interaction terms 'Im-vs-Co x Ti', 'Lo x Im-vs-Co x Ti', and 'Co(Lo) x Ti' (PERMANOVA; Table 3.2).

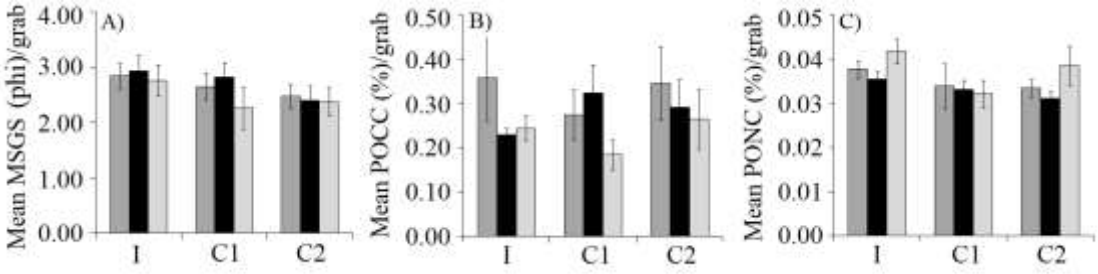


**Figure 3.2** Mean values ($\pm$SE) per grab of: (a) mean sediment grain size (MSGS) (phi), (b) percent organic carbon content (POCC), and (c) percent organic nitrogen content (PONC) recorded at the impacted and control plots in the years 2003 (dark grey bars), 2004 (black bars) and 2005 (light grey bars). I = impacted plot, C1 = 'Control 1' plot, C2 = 'Control 2' plot

**Table 3.2** Results of three-factor univariate asymmetrical PERMANOVA for sediment physico-chemical variables. Df = Degrees of freedom, MSGS = mean sediment grain size (phi), POCC = percent organic carbon content, PONC = percent organic nitrogen content, F-Ratio Nom = F-Ratio Nominator, F-Ratio Denom = F-Ratio Denominator, RES = Residual, ns = not significant, * = $p < 0.05$, *** = $p < 0.001$

| Source of Variation | df | MSGS | POCC | PONC | F-Ratio Nom | F-Ratio Denom |
|---|---|---|---|---|---|---|
| Location = Lo | 2 | *** | ns | * | Lo + Co(Lo) x Ti | Co(Lo) + Lo x Ti |
| Impact-vs-Control = Im-vs-Co | 1 | ns | ns | ns | Im-vs-Co + Lo x Im-vs-Co x Ti | Lo x Im-vs-Co + Im-vs-Co x Ti |
| Time = Ti | 2 | ns | ns | ns | Ti + Lo x Im-vs-Co x Ti | Im-vs-Co x Ti + Lo x Ti |
| Control(Lo) = Co(Lo) | 3 | ns | ns | ns | Co(Lo) | Co(Lo) x Ti |
| Lo x Im-vs-Co | 2 | * | * | ns | Lo x Im-vs-Co | Lo x Im-vs-Co x Ti |
| Lo x Ti | 4 | ns | ns | ns | Lo x Ti | Co(Lo) x Ti |
| Im-vs-Co x Ti | 2 | ns | ns | ns | Im-vs-Co x Ti | Lo x Im-vs-Co x Ti |
| Co(Lo) x Ti | 6 | ns | ns | ns | Co(Lo) x Ti | RES: Co(Lo) x Ti |
| Lo x Im-vs-Co x Ti | 4 | ns | ns | ns | Lo x Im-vs-Co x Ti | RES: Lo x Im-vs-Co x Ti |
| RES: Lo x Im-vs-Co x Ti | 18 | | | | | |
| RES: Co(Lo) x Ti | 36 | | | | | |
| Total | 80 | | | | | |

### 3.3.1(ii) Macroinvertebrate assemblages

A total of 19,293 individuals from 28 polychaete families, 1,920 individuals from 79 mollusc families, 6,137 amphipod individuals from 25 families, and 992 individuals from 23 decapod families, were collected. SIMPER showed that the three most important taxa contributing to the similarity of benthic assemblages in terms of abundance within the impacted plot, made up circa half (c. 49%) of the total polychaete abundances, and more than half of the total mollusc (c. 58%) and amphipod (c. 68%) abundances, while taxa showed lower dominance at the control plots, and the identity of some of the top contributing polychaete and mollusc taxa differed (see Table 3.3). Decapod assemblages were numerically dominated (c. 70-79%) by a single taxon (Paguridae) at both impacted and control plots (Table 3.3). The average similarity for mollusc (c. 11-12%) and decapod (c. 8-19%) assemblages in terms of abundance within impacted and control plots was low compared to that recorded for polychaete (c. 42-50%) and amphipod (c. 24-44%) assemblages (Table 3.3).

The polychaete and amphipod assemblage composition differed by more than half (57% and 69%, respectively) between the impacted and control plots, and a high

**Table 3.3** Results of SIMPER analysis calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data for : polychaetes, molluscs, amphipods, and decapods; showing the top three contributing families (in terms of abundance) to the similarity and dissimilarity of benthic assemblages recorded respectively within and between impact and control plots. Avg Sim (%) = Average Similarity (%), Cum Contrib (%) = Cumulative Contribution (%), Avg Dissim (%) = Average Dissimilarity (%), Contrib (%) = Contribution (%)

| | Impact | | | Control | | |
|---|---|---|---|---|---|---|
| | Avg Sim (%) | Family | Cum Contrib (%) | Avg Sim (%) | Family | Cum Contrib (%) |
| Polychaetes | 41.80 | Paraonidae | 21.83 | 50.04 | Paraonidae | 17.48 |
| | | Capitellidae | 37.31 | | Maldanidae | 32.57 |
| | | Glyceridae | 48.98 | | Glyceridae | 44.37 |
| Molluscs | 11.94 | Solemyidae | 33.26 | 10.52 | Carditidae | 14.50 |
| | | Nuculanidae | 49.13 | | Veneridae | 27.19 |
| | | Cerithiidae | 58.03 | | Cerithiidae | 36.29 |
| Amphipods | 24.15 | Urothoidae | 36.75 | 43.81 | Urothoidae | 19.60 |
| | | Corophiidae | 53.13 | | Corophiidae | 37.42 |
| | | Lysianassidae | 68.01 | | Lysianassidae | 54.04 |
| Decapods | 7.65 | Paguridae | 70.43 | 19.37 | Paguridae | 78.92 |
| | | - | - | | - | - |
| | | - | - | | - | - |

| | Impact-vs-Control | | | | |
|---|---|---|---|---|---|
| | Avg Dissim (%) | | Average Abundance | | |
| | | Family | Impact | Control | Contrib (%) |
| Polychaetes | 57.29 | Capitellidae | 1.78 | 0.84 | 9.42 |
| | | Maldanidae | 0.84 | 1.48 | 6.02 |
| | | Sabellidae | 0.43 | 0.97 | 5.32 |
| Molluscs | 90.66 | Solemyidae | 0.46 | 0.13 | 7.34 |
| | | Nuculanidae | 0.29 | 0.18 | 5.99 |
| | | Cerithiidae | 0.34 | 0.30 | 5.82 |
| Amphipods | 69.32 | Urothoidae | 0.95 | 1.10 | 10.35 |
| | | Corophiidae | 0.69 | 1.08 | 10.22 |
| | | Lysianassidae | 0.60 | 0.99 | 9.33 |
| Decapods | 88.37 | Paguridae | 0.41 | 0.77 | 34.55 |
| | | Processidae | 0.13 | 0.14 | 10.30 |
| | | Galatheidae | 0.16 | 0.23 | 10.28 |

dissimilarity was recorded for mollusc and decapod assemblages (91% and 88%, respectively; Table 3.3). The polychaete taxa contibuting most to dissimilarity were Capitellidae, which were more abundant at the impacted plots, and Maldanidae and Sabellidae, which were less abundant at the impacted plots, compared to the control

plots (Table 3.3). The three amphipod taxa (Urothoidae, Corophiidae, and Lysianassidae) and decapod taxa (Paguridae, Processidae, and Galatheidae), contributing most to the dissimilarity in assemblage composition between the impacted and control plots, were less abundant at the impacted plots compared to the control plots, while mollusc taxa (Solemyidae, Nuculanidae, and Cerithiidae) contibuting most to the dissimilarity, were more abundant at the impacted plots compared to the control plots (Table 3.3).

Univariate PERMANOVA indicated no significant differences for 'Im-vs-Co x Ti' in the polychaete, amphipod, mollusc, and decapod indicator families (Table 3.4). Despite the lack of significant differences between impacted and control plots over time, an elevated abundance of Capitellidae (Polychaeta) was recorded at the impacted plot compared to the two control plots, while Maldanidae (Polychaeta), Carditidae (Mollusca), Corophiidae (Amphipoda) and Paguridae (Decapoda) showed the opposite pattern (Figure 3.3).

PERMANOVA indicated a significant difference for 'Lo x Im-vs-Co x Ti' in the abundance of Capitellidae ($p < 0.05$), Maldanidae ($p < 0.0001$), Sabellidae ($p < 0.05$) and Processidae ($p < 0.01$), and for 'Co(Lo) x Ti' in the abundance of Sabellidae (Polychaeta) ($p < 0.01$) (Table 3.4). PERMANOVA also indicated a significant difference for 'Si(Lo x Im-vs-Co x Ti)' in the abundance of Maldanidae ($p < 0.05$), Corophiidae ($p < 0.001$), Lysianassidae ($p < 0.01$) and Paguridae ($p < 0.0001$), and for 'Si(Co(Lo) x Ti)' in the abundance of Capitellidae ($p < 0.0001$), Maldanidae ($p < 0.0001$), Sabellidae ($p < 0.05$), Solemyidae ($p < 0.01$) and Paguridae ($p < 0.0001$) (Table 3.4). *A posteriori* PERMANOVA indicated no significant difference for 'Im-vs-Co x Ti', 'Lo x Im-vs-Co x Ti', and 'Co(Lo) x Ti', in the abundance of Nuculanidae (Mollusca), while 'Si(Co(Lo) x Ti)' was significant ($p < 0.001$) (Table 3.4).

Number of families and Shannon-Wiener diversity over time were similar for polychaetes, molluscs and amphipods, and appeared low at the impacted plot compared to the two control plots. For decapods, the number of families and Shannon-Wiener diversity were similar over time between impacted and control plots (Figure 3.4). Values of mean polychaete/amphipod (BOPA-Fish farming) index changed at the

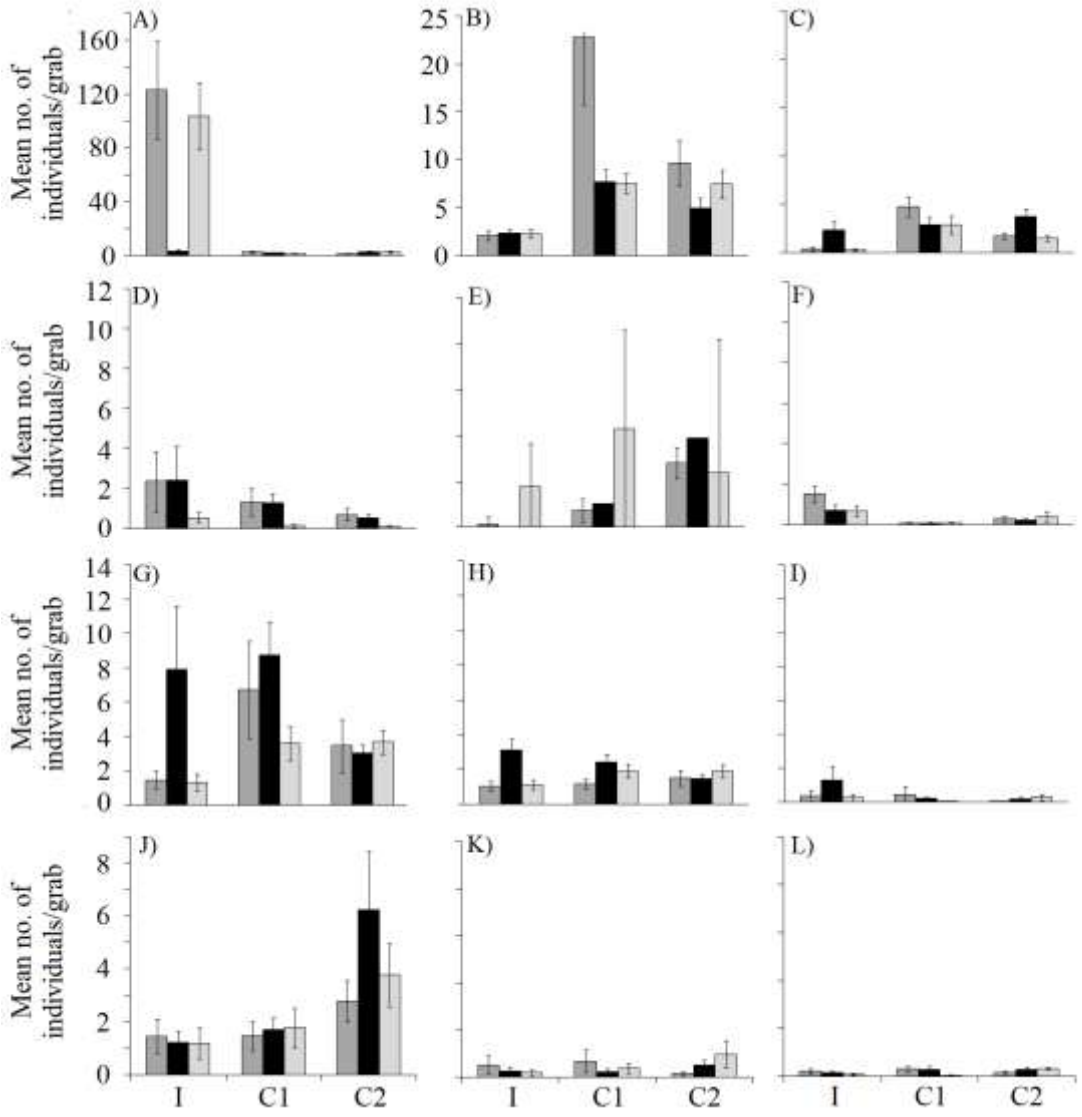


**Figure 3.3** Mean values (±SE) per grab of number of individuals of the indicator taxa: (a) Capitellidae, (b) Maldanidae and (c) Sabellidae (Polychaeta); (d) Cerithiidae, (e) Carditidae and (f) Solemyidae (Mollusca); (g) Corophiidae, (h) Urothoidae and (i) Lysianassidae (Amphipoda); (j) Paguridae, (k) Galatheidae and (l) Processidae (Decapoda), recorded at the impacted and control plots in the years 2003 (dark grey bars), 2004 (black bars) and 2005 (light grey bars). I = impacted plot, C1 = 'Control 1' plot, C2 = 'Control 2' plot

impacted plot from 'Poor'/'Moderate' to 'Good' EQS, while 'Good' EQS was indicated at the two control plots over time (Figure 3.4).

However, PERMANOVA indicated no significant difference for 'Im-vs-Co x Ti' in the number of families and Shannon-Wiener diversity of polychaetes, molluscs, amphipods and decapods, and in the polychaete/amphipod index, while 'Lo x Im-vs-Co x Ti' was significant for the Shannon-Wiener diversity of polychaetes ($p <$

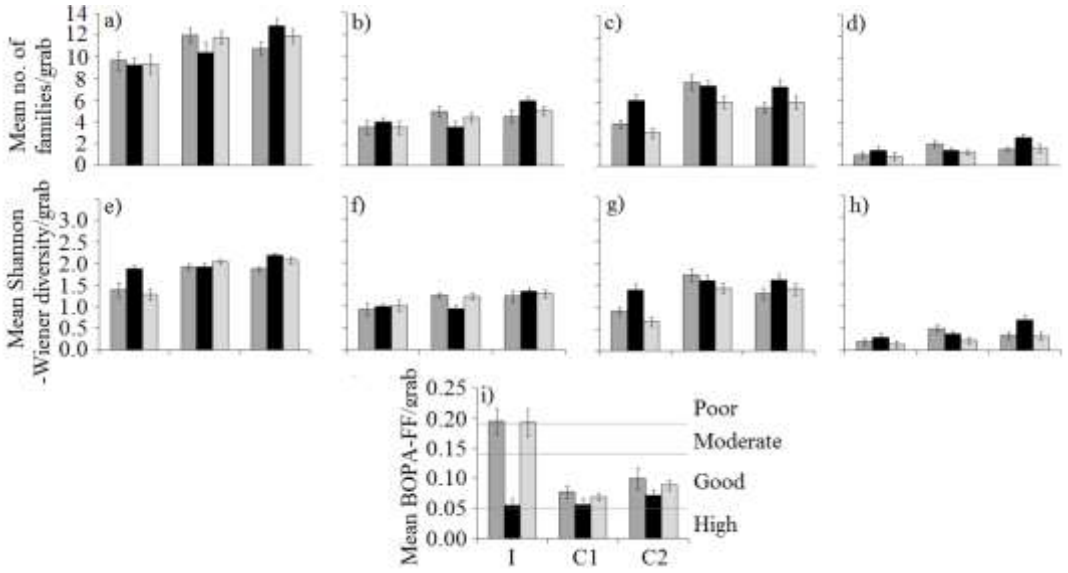


**Figure 3.4** Mean values (±SE) per grab of: (a-d) total number of families and (e-h) Shannon-Wiener diversity of (a, e) polychaetes, (b, f) molluscs, (c, g) amphipods, and (d, h) decapods, and (i) the polychaete/amphipod index, recorded at the impacted and control plots in the years 2003 (dark grey bars), 2004 (black bars) and 2005 (light grey bars). I = impacted plot, C1 = 'Control 1' plot, C2 = 'Control 2' plot

0.01), number of amphipod families ($p < 0.05$) and Shannon-Wiener diversity of amphipods ($p < 0.01$) (Table 3.4).

PERMANOVA indicated a significant difference for 'Co(Lo) x Ti' in the number of polychaete families ($p < 0.01$), number of amphipod families, and Shannon-Wiener diversity of amphipods ($p < 0.001$) (Table 3.4). PERMANOVA also indicated a significant difference for 'Si(Lo x Im-vs-Co x Ti)' in the number of polychaete families ($p < 0.001$), number of decapod families, Shannon-Wiener diversity of decapods ($p < 0.001$), and polychaete/amphipod index ($p < 0.01$); and for 'Si(Co(Lo) x Ti)' in the number of families and Shannon-Wiener diversity of polychaetes ($p_{NFa} < 0.01$; $p_{ShW} < 0.0001$), amphipods and decapods ($p < 0.05$), in the number of mollusc families ($p < 0.01$), and in the polychaete/amphipod index ($p < 0.01$) (Table 3.4).

### 3.3.2 Multivariate data analyses

#### 3.3.2(i) Sediment physico-chemical attributes

PCO ordination of MSGS, POCC and PONC of the sediment explained 43.3% of the total variation in the sediment physico-chemical data, and showed separation of

**Table 3.4** Results of four-factor univariate asymmetrical PERMANOVA for attributes of the macrobenthic assemblages. Df = Degrees of freedom, RES = Residual, NI = number of individuals, NFa = number of families, ShW = Shannon-Wiener diversity, Polychaete indicator taxon 1 = Capitellidae, 2 = Maldanidae, 3 = Sabellidae; Mollusc indicator taxon 1 = Cerithiidae, 2 = Carditidae, 3 = Solemyidae; Amphipod indicator taxon 1 = Corophiidae, 2 = Urothoidae, 3 = Lysianassidae; Decapod indicator taxon 1 = Paguridae, 2 = Galatheidae, 3 = Processidae; P/A = Polychaete/Amphipod ratio; Nuc = Nuculanidae; ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

| | | Polychaetes | | | | | Molluscs | | | | | Amphipods | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | df | NI 1 | N1 2 | NI 3 | NFa | ShW | NI 1 | N1 2 | NI 3 | NFa | ShW | NI 1 | N1 2 | NI 3 | NFa | ShW |
| Location = Lo | 2 | ns | ns | ns | * | *** | ns | ns | * | ** | * | ns | ns | ns | ns | ns |
| Impact-vs-Control = Im-vs-Co | 1 | ns | ns | * | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | * | ns |
| Time = Ti | 2 | ns | ns | ns | ns | ns | * | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Control(Lo) = Co(Lo) | 3 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Lo x Im-vs-Co | 2 | ns | ns | ns | ns | ns | ns | ns | * | ** | * | ns | ns | ns | ns | ns |
| Lo x Ti | 4 | | ns | ns | ns | ns | ns | ns | * | ns | ns | ns | ns | ns | ns | ns |
| Im-vs-Co x Ti | 2 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Co(Lo) x Ti | 6 | ns | ns | ** | ** | ns | ns | ns | ns | ns | ns | ns | ns | ns | *** | *** |
| Lo x Im-vs-Co x Ti | 4 | * | **** | * | ns | ** | ns | * | ns | ns | ns | ns | ns | ns | * | ** |
| Site(Lo x Im-vs-Co x Ti) = Si(Lo x Im-vs-Co x Ti) | 18 | ns | * | ns | *** | ns | ns | ns | ns | ns | ns | *** | ns | ** | ns | ns |
| Site(Co(Lo) x Ti) = Si(Co(Lo) x Ti) | 36 | **** | **** | * | ** | **** | ns | ns | ** | ** | ns | ns | ns | ns | * | * |
| RES: Si(Lo x Im-vs-Co x Ti) | 54 | | | | | | | | | | | | | | | |
| RES: Si(Co(Lo) x Ti) | 108 | | | | | | | | | | | | | | | |
| Total | 242 | | | | | | | | | | | | | | | |

**Table 3.4** Continued

| | | Decapods | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Source of Variation | df | NI 1 | N12 | NI3 | NFa | ShW | Nuc | P/A |
| Location = Lo | 2 | ns | ns | * | ns | ns | ns | ns |
| Impact-vs-Control = Im-vs-Co | 1 | ns | ns | ns | ns | ns | ns | ns |
| Time = Ti | 2 | ns | ns | ns | ns | ns | ns | ns |
| Control(Lo) = Co(Lo) | 3 | * | ns | ns | ns | ns | ***** | ns |
| Lo x Im-vs-Co | 2 | * | ns | ns | ns | ns | ****** | ***** |
| Lo x Ti | 4 | ns | ns | ns | ns | ns | ns | ns |
| Im-vs-Co x Ti | 2 | ns | ns | ns | ns | ns | ns | ns |
| Co(Lo) x Ti | 6 | ns | ns | ns | ns | ns | ns | ns |
| Lo x Im-vs-Co x Ti | 4 | ns | ns | ** | ns | ns | ns | ns |
| Site(Lo x Im-vs-Co x Ti) = Si(Lo x Im-vs-Co x Ti) | 18 | **** | ns | ns | *** | *** | ns | ****** |
| Site(Co(Lo) x Ti) = Si(Co(Lo) x Ti) | 36 | **** | ns | ns | * | * | ******** | ****** |
| RES: Si(Lo x Im-vs-Co x Ti) | 54 | | | | | | | |
| RES: Si(Co(Lo) x Ti) | 108 | | | | | | | |
| Total | 242 | | | | | | | |

**Table 3.4** Continued

| Source of Variation | F-ratio Nominator | F-ratio Denominator |
|---|---|---|
| Location = Lo | Lo + Co(Lo) x Ti | Co(Lo) + Lo x Ti |
| Impact-vs-Control = Im-vs-Co | Im-vs-Co + Lo x Im-vs-Co x Ti | Lo x Im-vs-Co + Im-vs-Co x Ti |
| Time = Ti | Ti + Lo x Im-vs-Co x Ti | Im-vs-Co x Ti + Lo x Ti |
| Control(Lo) = Co(Lo) | Co(Lo) | Co(Lo) x Ti |
| Lo x Im-vs-Co | Lo x Im-vs-Co | Lo x Im-vs-Co x Ti |
| Lo x Ti | Lo x Ti | Co(Lo) x Ti |
| Im-vs-Co x Ti | Im-vs-Co x Ti | Lo x Im-vs-Co x Ti |
| Co(Lo) x Ti | Co(Lo) x Ti | Si(Co(Lo) x Ti) |
| Lo x Im-vs-Co x Ti | Lo x Im-vs-Co x Ti | Si(Lo x Im-vs-Co x Ti) |
| Site(Lo x Im-vs-Co x Ti) = Si(Lo x Im-vs-Co x Ti) | Si(Lo x Im-vs-Co x Ti) | RES: Si(Lo x Im-vs-Co x Ti) |
| Site(Co(Lo) x Ti) = Si(Co(Lo) x Ti) | Si(Co(Lo) x Ti) | RES: Si(Co(Lo) x Ti) |
| RES: Si(Lo x Im-vs-Co x Ti) | | |
| RES: Si(Co(Lo) x Ti) | | |
| Total | | |

sediment samples between the three years of sampling, but no grouping of sediment samples between impacted and control plots over time was evident (Figure 3.5).

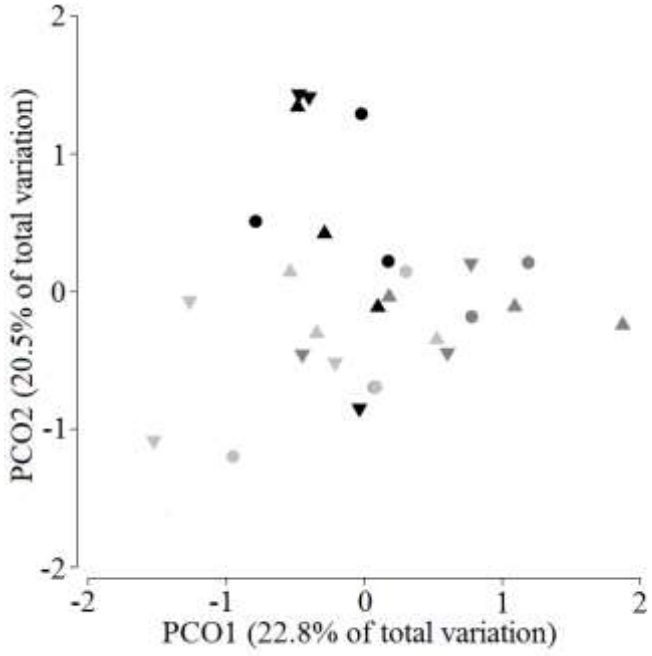


**Figure 3.5** PCO plot calculated from a Euclidean similarity matrix of normalised sediment physico-chemical data, i.e.: mean sediment grain size, percent organic carbon content, and percent organic nitrogen content, recorded at the impacted (circle), 'Control 1' (triangle) and 'Control 2' (inverted triangle) plots in the year 2003 (dark grey), 2004 (black) and 2005 (light grey).

Multivariate PERMANOVA indicated that the square root estimate of variation for 'Im-vs-Co x Ti' (-0.06714), 'Lo x Im-vs-Co x Ti' (-0.56031), and 'Co(Lo) x Ti' (0.15727), as components of variation in sediment physico-chemical data, were negative or small (Table 3.5). Both PERMANOVA and PERMDISP tests indicated no significant difference for 'Im-vs-Co x Ti' in sediment physico-chemical data, while 'Lo x Im-vs-Co x Ti' ($p_{PERMDISP} < 0.01$) and 'Co(Lo) x Ti' ($p_{PERMDISP} < 0.05$) were significant (Table 3.5).

### 3.3.2(ii) Macroinvertebrate assemblages

PCO ordination explained less than a third of the total variation in the assemblage composition of polychaete (29.1%), mollusc (21.4%) and amphipod (26.6%) data, while PCO ordination of decapod data explained only 17.3% of the total variation in the assemblage composition (Figure 3.6). Polychaete, mollusc, amphipod and decapod

**Table 3.5** Results of three-factor multivariate asymmetrical PERMANOVA and PERMDISP tests calculated from a Euclidean similarity matrix of normalised values of mean sediment grain size, percent organic carbon content, and percent organic nitrogen content. Df = Degrees of freedom, RES= Residual, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$

| | | PERMANOVA | | PERMDISP |
|---|---|---|---|---|
| Source of Variation | df | Sq Rt Var | p-value | p-value |
| Location = Lo | 2 | 0.7739 | * | ns |
| Impact-vs-Control = Im-vs-Co | 1 | -0.2526 | ns | ns |
| Time = Ti | 2 | 0.3440 | ns | ns |
| Control(Lo) = Co(Lo) | 3 | -0.1391 | ns | * |
| Lo x Im-vs-Co | 2 | 0.8430 | * | * |
| Lo x Ti | 4 | -0.4043 | ns | ns |
| Im-vs-Co x Ti | 2 | -0.0671 | ns | ns |
| Co(Lo) x Ti | 6 | 0.1573 | ns | * |
| Lo x Im-vs-Co x Ti | 4 | -0.5603 | ns | ** |
| RES: Lo x Im-vs-Co x Ti | 18 | 0.0636 | | |
| RES: Co(Lo) x Ti | 36 | 1.4570 | | |
| Total | 80 | | | |

data showed no clear grouping of samples between the impacted and control plots over time (Figure 3.6).

Multivariate PERMANOVA indicated that values of the square root estimate of variation for 'Im-vs-Co x Ti' and 'Co(Lo) x Ti' for polychaetes (6.231 and 6.534), molluscs (0.9539 and -9.493), amphipods (6.352 and 8.253) and decapods (-0.1415 and -8.367), were small or negative, while the square root estimate of variation for 'Lo x Im- vs-Co x Ti' was higher for molluscs (16.04) and decapods (11.76) compared to polychaetes (2.763) and amphipods (1.741) (Table 3.6). On the other hand, PERMANOVA showed that the square root estimate of variation for 'RES: Si(Co(Lo) x Ti)' as a component of variation in the assemblage composition of polychaetes (26.74), molluscs (55.72), amphipods (32.12) and decapods (56.17), was large (Table 3.6).

PERMDISP indicated a significant difference for 'Im-vs-Co x Ti' in the assemblage composition of polychaetes ($p < 0.01$), amphipods and decapods ($p < 0.001$), while PERMANOVA did not (Table 3.6). Dispersion of samples of polychaete, amphipod and decapod data over time was significantly higher at the impacted plot compared to the control plots (Figure 3.6). Both PERMANOVA and PERMDISP tests indicated no significant

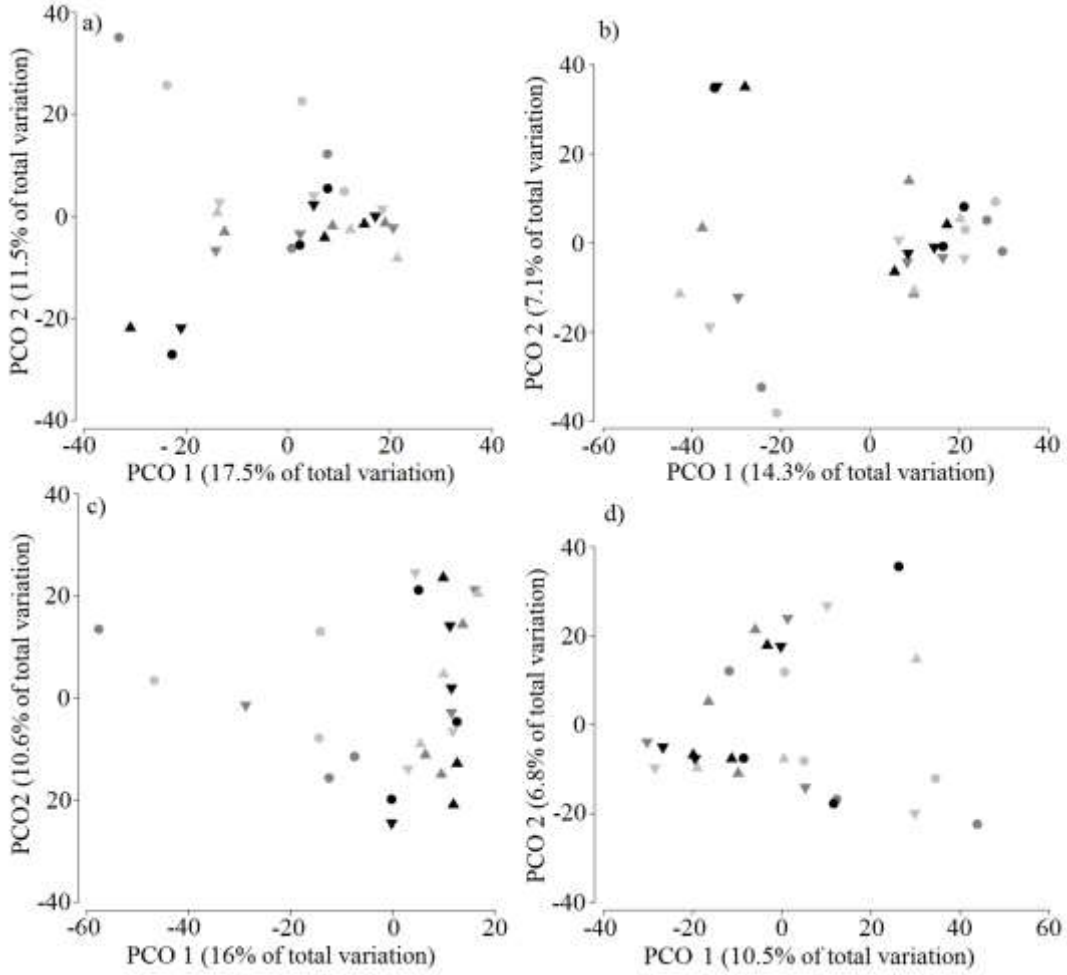


**Figure 3.6** PCO plots calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data recorded from the putative impacted plots (circle) and from the 'Control 1' (upward triangle) and 'Control 2' (inverted triangle) plots in the year 2003 (dark grey), 2004 (black) and 2005 (light grey), for (a) polychaetes, (b) molluscs, (c) amphipods and (d) decapods.

difference in the assemblage composition of molluscs over time between impacted and control plots ('Im-vs-Co x Ti') (Table 3.6).

PERMDISP tests indicated significant differences for 'Lo x Im-vs-Co x Ti' and 'Co(Lo) x Ti' in the assemblage composition of polychaetes ($p_{\text{Lo x Im-vs-Co x Ti}} < 0.001$; $p_{\text{Co(Lo) x Ti}} < 0.01$), molluscs, amphipods and decapods ($p_{\text{Lo x Im-vs-Co x Ti}} < 0.001$; $p < {}_{\text{Co(Lo) x Ti}}\ 0.001$), while PERMANOVA did not (Table 3.6). Significant differences were also indicated for 'Si(Lo x Im-vs-Co x Ti)' in polychaete, mollusc, amphipod ($p_{\text{PERMDISP}} < 0.001$) and decapod ($p_{\text{PERMDISP}} < 0.01$) assemblages, and for 'Si(Co(Lo) x Ti)' in polychaete (p

**Table 3.6** Results of four-factor multivariate asymmetrical PERMANOVA and PERMDISP tests calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data for: polychaetes, molluscs, amphipods and decapods. Variables included in each analysis are fourth-root transformed family abundance of the macroinvertebrate faunal group. Df = Degrees of freedom, Sqr Rt Var = Square Root Estimate of Component of Variation, RES = Residual, ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001

| | | Polychaetes | | | Molluscs | | | Amphipods | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | PERMANOVA | | PERMDISP | PERMANOVA | | PERMDISP | PERMANOVA | | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value | Sq Rt Var | p-value | p-value | Sq Rt Var | p-value | p-value |
| Location = Lo | 2 | 17.11 | ns | *** | 23 | ns | *** | 12.38 | ns | ns |
| Impact-vs-Control = Im-vs-Co | 1 | 10.78 | ns | *** | 10.2 | ns | ns | 6.184 | ns | *** |
| Time = Ti | 2 | -3.598 | ns | ns | 1.69 | ns | ns | 4.087 | ns | *** |
| Control(Lo) = Co(Lo) | 3 | 3.173 | ns | *** | 11.64 | ns | *** | 9.292 | ns | *** |
| Lo x Im-vs-Co | 2 | 5.298 | ns | *** | 0.799 | ns | *** | 0.8245 | ns | *** |
| Lo x Ti | 4 | 14.8 | ns | ** | 17.18 | ns | *** | 11.73 | ns | *** |
| Im-vs-Co x Ti | 2 | 6.231 | ns | ** | 0.9539 | ns | ns | 6.352 | ns | *** |
| Co(Lo) x Ti | 6 | 6.534 | ns | *** | -9.493 | ns | *** | 8.253 | ns | *** |
| Lo x Im-vs-Co x Ti | 4 | 2.763 | ns | *** | 16.038 | ns | *** | 1.741 | ns | *** |
| Site(Lo x Im-vs-Co x Ti) = Si(Lo x Im-vs-Co x Ti) | 18 | 0.576 | ns | *** | 1.475 | ns | *** | 1.694 | ns | *** |
| Site(Co(Lo) x Ti) = Si(Co(Lo) x Ti) | 36 | 13.85 | * | *** | 18.34 | ns | ns | 18.81 | ** | ns |
| RES: Si(Lo x Im-vs-Co x Ti) | 54 | 1.752 | | | -1.325 | | | 5.927 | | |
| RES: Si(Co(Lo) x Ti) | 108 | 26.74 | | | 55.72 | | | 32.12 | | |
| Total | 242 | | | | | | | | | |

**Table 3.6** Continued

| | | Decapods | | |
|---|---|---|---|---|
| | | PERMANOVA | | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value |
| Location = Lo | 2 | 9.486 | ns | * |
| Impact-vs-Control = Im-vs-Co | 1 | 9.1 | ns | *** |
| Time = Ti | 2 | -4.156 | ns | * |
| Control(Lo) = Co(Lo) | 3 | 11.61 | ns | *** |
| Lo x Im-vs-Co | 2 | 1.355 | ns | *** |
| Lo x Ti | 4 | 8.246 | ns | ** |
| Im-vs-Co x Ti | 2 | -0.1415 | ns | *** |
| Co(Lo) x Ti | 6 | -8.367 | ns | *** |
| Lo x Im-vs-Co x Ti | 4 | 11.76 | ns | *** |
| Site(Lo x Im-vs-Co x Ti) = Si(Lo x Im-vs-Co x Ti) | 18 | -2.085 | ns | ** |
| Site(Co(Lo) x Ti) = Si(Co(Lo) x Ti) | 36 | 16.32 | ns | *** |
| RES: Si(Lo x Im-vs-Co x Ti) | 54 | 3.549 | | |
| RES: Si(Co(Lo) x Ti) | 108 | 56.17 | | |
| Total | 242 | | | |

$p_{PERMANOVA} < 0.05$, $p_{PERMDISP} < 0.001$), amphipod ($p_{PERMANOVA} < 0.01$) and decapod ($p_{PERMDISP} < 0.001$) assemblages (Table 3.6).

### 3.3.3 Relationship between sediment attributes and macroinvertebrates

BEST analysis showed that the POCC of the sediment was significantly correlated with the observed variation in Capitellidae abundance ($p < 0.05$, $\rho = 0.349$), number of families ($p = 0.05$, $\rho = 0.209$) and Shannon-Wiener diversity ($p = 0.05$, $\rho = 0.228$) of amphipods, and assemblage composition of decapods ($p < 0.05$, $\rho = 0.342$), while a combination of MSGS and POCC of the sediment was significantly correlated with the abundance of Paguridae ($p < 0.05$, $\rho = 0.428$) and Shannon-Wiener diversity of decapods ($p < 0.05$, $\rho = 0.356$), at the impacted plot (Table 3.7).

At the control plots, there was significant correlation between the MSGS and the Shannon-Wiener diversity of polychaetes ($p < 0.05$, $\rho = 0.145$); between a combination of the POCC and PONC of the sediment, and the abundance of Capitellidae ($p < 0.05$, $\rho = 0.211$) and Paguridae ($p < 0.01$, $\rho = 0.263$); between a combination of MSGS and POCC, and the number of polychaete families ($p < 0.01$, $\rho = 0.233$) and the assemblage composition of polychaetes ($p < 0.01$, $\rho = 0.410$) and amphipods ($p < 0.01$, $\rho = 0.289$); between a combination of MSGS and PONC of the sediment, and the assemblage composition of molluscs ($p < 0.01$, $\rho = 0.217$); and between a combination of MSGS, POCC and PONC of the sediment, and the polychaete/amphipod index ($p < 0.05$, $\rho = 0.195$) (Table 3.7).

**Table 3.7** Results of BEST analysis showing the environmental variable or combination thereof, that best explained the recorded variation in attributes of the macroinvertebrate assemblages recorded overall at the impacted and control plots. Level of significance set at 0.05. A Euclidean similarity matrix was used for univariate biotic data, while a Bray Curtis similarity matrix of fourth-root transformed family abundance data was used for multivariate analyses. Dep Var = dependent variable, ρ-value = Spearman's rank correlation coefficient, Best Exp Var = Best Explanatory Variable, NI = number of individuals, NFa = number of families, ShW = Shannon-Wiener diversity; Polychaete indicator taxon 1 = Capitellidae, 2 = Maldanidae, 3 = Sabellidae; Mollusc indicator taxon 1 = Cerithiidae, 2 = Carditidae, 3 = Solemyidae; Amphipod indicator taxon 1 = Corophiidae, 2 = Urothoidae, 3 = Lysianassidae; Decapod indicator taxon 1 = Paguridae, 2 = Galatheidae, 3 = Processidae; P/A = Polychaete/Amphipod ratio, AsC = Assemblage Composition, MSGS = mean sediment grain size, POCC = percent organic carbon content, PONC = percent organic nitrogen content, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$

| | | Impact | | | Controls | | |
|---|---|---|---|---|---|---|---|
| | Dep Var | ρ-value | p-value | Best Exp Var | ρ-value | p-value | Best Exp Var |
| Polychaetes | NI 1 | 0.349 | * | POCC | 0.211 | * | POCC, PONC |
| | NI 2 | 0.025 | ns | POCC | 0.023 | ns | MSGS |
| | NI 3 | 0.171 | ns | MSGS, POCC, PONC | 0.019 | ns | PONC |
| | NFa | 0.233 | ns | MSGS, POCC | 0.233 | ** | MSGS, OCC |
| | ShW | 0.139 | ns | POCC | 0.145 | * | MSGS |
| | AsC | 0.057 | ns | PONC | 0.410 | ** | MSGS, POCC |
| Molluscs | NI 1 | 0.271 | ns | POCC | 0.130 | ns | MSGS, POCC |
| | NI 2 | 0.097 | ns | MSGS | -0.022 | ns | MSGS |
| | NI 3 | 0.136 | ns | MSGS, POCC | 0.073 | ns | MSGS, PONC |
| | NFa | 0.166 | ns | MSGS, POCC | 0.110 | ns | MSGS |
| | ShW | 0.066 | ns | MSGS | 0.098 | ns | MSGS |
| | AsC | 0.026 | ns | PONC | 0.217 | ** | MSGS, PONC |
| Amphipods | NI 1 | 0.199 | ns | MSGS, POCC, PONC | 0.029 | ns | POCC |
| | NI 2 | -0.098 | ns | PONC | -0.012 | ns | PONC |
| | NI 3 | 0.213 | ns | MSGS, POCC | 0.041 | ns | PONC |
| | NFa | 0.209 | * | POCC | 0.024 | ns | PONC |
| | ShW | 0.228 | * | POCC | 0.148 | ns | POCC |
| | AsC | 0.249 | ns | POCC | 0.289 | ** | MSGS, POCC |
| Decapods | NI 1 | 0.428 | * | MSGS, POCC | 0.263 | ** | POCC, PONC |
| | NI 2 | 0.256 | ns | POCC | 0.171 | ns | PONC |
| | NI 3 | 0.230 | ns | POCC | 0.029 | ns | PONC |
| | NFa | 0.303 | ns | MSGS, POCC | 0.103 | ns | PONC |
| | ShW | 0.356 | * | MSGS, POCC | 0.041 | ns | PONC |
| | AsC | 0.342 | * | POCC | -0.021 | ns | MSGS |
| P/A index | | 0.122 | ns | POCC, PONC | 0.195 | * | MSGS, POCC, PONC |

## 3.4 Discussion

The present work assessed data from three different ABT farms and six control areas collected over three years of farming operations; this is the first time that a study of the influence of tuna penning on the marine environment incorporates such a design. Present results indicate that change in soft bottom habitat macroinvertebrate assemblages resulting from the influence of the tuna farms was consistent across the polychaete and amphipod faunal groups when used as indicators of change, while the molluscs and decapods showed low similarity in terms of abundance within plots, which made them poor indicators of differences in macroinvertebrate assemblages among impacted and control areas.

The seabed in the vicinity of tuna farms is subjected to high sediment organic loading resulting from the high biomass of farmed fish and an overfeeding regime aimed at reaching elevated oil contents in the captive fish (Aguado-Giménez *et al.*, 2006). Accumulations of uneaten feed-fish on the seabed below tuna cages (Holmer *et al*., 2008; Mangion *et al.*, 2014) form a decomposing mass of organic matter that enhances microbial activity, leading to low sediment oxygen conditions (Giles, 2008). Such conditions are tolerated only by some opportunistic species, and result in reduced macrofaunal diversity (Giles, 2008) in the vicinity of tuna cages during the fattening season (e.g. Mangion *et al*., 2014; Marin *et al.*, 2007; Vita & Marin, 2007). Capitellids are opportunistic polychaetes (Borja *et al*., 2000) that are notoriously abundant below fish farms (e.g. Dean, 2008; Karakassis *et al*., 2000; Vita & Marin, 2007), and would characteristically dominate benthic macroinvertebrate assemblages near a source of organic enrichment (Pearson & Rosenberg, 1978) and hence act as good indicators of organic pollution. On the other hand, malacostracan crustaceans, gastropods and bivalves are generally sensitive to low sediment oxygen levels (Sanz-Lázaro & Marin, 2011).

Studies have shown that polychaetes used as surrogates for macrobenthic diversity (Olsgard, Brattegard, & Holthe, 2003; Olsgard & Somerfield, 2000) reflect patterns of the whole macrobenthic community from the species to the order level along both natural (Wlodarska-Kowalczuk & Kedra, 2007) and pollution (Olsgard & Somerfield, 2000) disturbance gradients. Wlodarska-Kowalczuk and Kedra (2007) contended that

the distribution pattern of the whole macrobenthic community along natural disturbance gradients does not persist when considering only crustaceans or molluscs. The performance of bioindicators also differs depending on habitat and the environmental regime (Wlodarska-Kowalczuk & Kedra, 2007). In the present study, polychaetes had the highest total abundance, followed by amphipods. Polychaetes, amphipods and decapods had a similar diversity, while molluscs had the highest diversity. However, both molluscs and decapods had a low total abundance, which limits their use in environmental monitoring studies. The results of multivariate analyses did not detect any significant effect of tuna penning at the impacted plots on mollusc assemblages, which may be attributed to the high variation in mollusc family abundance recorded at the smallest spatial scale (i.e., 100 m's). It is also worth noting that while decapods are scavengers and hence attracted by the presence of bulk decomposing organic material such as that found on the sediment below tuna cages, they are generally more mobile than the other considered taxa, which may further limit their usefulness as indicators. Therefore, polychaete and amphipod assemblages appear to be better indicators of change in macroinvertebrate assemblages resulting from the influence of tuna penning.

Polychaetes, which are found in all benthic habitats (Pocklington & Wells, 1992), are highly sensitive to different types of alteration of soft sediments such as organic loading (Tsutsumi *et al*., 2001). The use of polychaetes as environmental indicators is well established (e.g. Dean, 2008; Giangrande, Licciano, & Musco, 2005), including their use as indicators of fish farming influence on benthic habitats (e.g. Aguado-Giménez *et al.*, 2015; Martinez-Garcia *et al.*, 2013; Sutherland *et al*., 2007; Tomassetti & Porrello, 2005). The results of the multivariate analyses reported here indicate significantly higher dispersion of samples of the polychaete assemblage over time at the impacted plot compared to the two control plots, but no significant difference in the number of taxa and diversity of polychaetes between impacted and control plots was detected over time. These results contrast with previous studies that recorded a significant decrease in polychaete abundance, species richness and diversity in the vicinity of fish farms (Lee *et al*., 2006; Martinez-Garcia *et al.*, 2013). Martinez-Garcia *et al.* (2013) found that organic loading from fish farms sited over soft bottom habitats in temperate areas may be gauged by the presence of tolerant polychaete families, such as Capitellidae, while families sensitive to fish farm wastes and indicative of non-

polluted conditions include Maldanidae and Sabellidae. However, the usefulness of polychaete taxa as indicators of polluted conditions may vary geographically and temporally (Dean, 2008). In the present study, an elevated (albeit not significant) abundance of Capitellid polychaetes recorded over time from impacted stations compared to control stations, was significantly correlated to the sediment POCC, and supports the findings by Vita and Marin (2007). On the other hand, the present results indicate low abundance of Maldanidae and Sabellidae at the impacted plot compared to the control plots. In spite of no significant difference between plots over time, polychaete indicator taxa with known responses to fish farm wastes appear to be more suitable for evaluating the environmental influence of tuna penning compared to mollusc and amphipod indicator families (see below).

Crustaceans, which include amphipods, are an important group of benthic fauna in terms of abundance and diversity (Thomas, 1993). The sensitivity of amphipods to pollution is generally greater than that of other Crustacea (Dauvin, 1987; 1998), hence some workers (e.g. Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011) recommend the use of this taxon as a biological indicator of change potentially resulting from fish farming activities. The present results indicated significant correlation between the low (albeit not significant) number of families and diversity of amphipods, and the sediment POCC, and significantly high dispersion of samples of the amphipod assemblages, over time at the impacted plot compared to the two control plots. These results support findings from previous studies that recorded a decrease in abundance and species richness of amphipods in the vicinity of Mediterranean sea bream/sea bass fish farms (Fernandez-Gonzalez *et al*., 2013), and are suggestive of the usefulness of amphipods as a biological indicator to monitor the influence of tuna penning activities on benthic habitats. However, no significant difference in the abundance of amphipod indicator taxa over time was detected between impacted and control plots in the present study, although Corophiidae showed a low abundance at the impacted plot. Previous workers recorded a decrease in abundance of crustaceans (La Rosa, Mirto, Mazzola, & Danovaro, 2001; Fernandez-Gonzalez & Sanchez-Jerez, 2011) near Mediterranean sea bream/sea bass fish farms, and in the diversity of crustaceans in the vicinity of a Scottish salmon farm (Hall-Spencer & Bamber, 2007).

The polychaete/amphipod ratio (BOPA) is a measure of the relative abundance of non-sensitive opportunistic species to sensitive species in organically enriched sediment (Dauvin & Ruellet, 2007; Gomez-Gesteira & Dauvin, 2000). BOPA "considers the total number of individuals in the samples, the frequency of opportunistic polychaetes, and the frequency of amphipods excluding the genus *Jassa*" (Dauvin & Ruellet, 2007, p. 215). The BOPA index has been used in assessing environmental impacts resulting from different activities (e.g. Andrade & Renaud, 2011; De-la-Ossa-Carretero, Del-Pilar-Ruso, Giménez-Casalduero, & Sánchez-Lizaso, 2009; Nikitik & Robinson, 2003), including fish farming (Aguado-Giménez *et al.*, 2015; Jahani *et al.*, 2012). Aguado-Giménez *et al*. (2015) recalculated the BOPA index by inclusion of polychaete families identified by Martinez-Garcia *et al*. (2013) to be tolerant to fish farm wastes, and defined the new BOPA-Fish Farming index. The present results show a higher ratio of opportunistic polychaete to sensitive amphipod taxon abundances at the impacted plot compared to the two control plots, but no significant difference in the polychaete/amphipod ratio was detected between the impacted plot and the two control plots, over time. Previously, Aguado-Giménez *et al*. (2015) found that BOPA poorly described the fish farming influence gradient since low amphipod abundance and tolerant polychaete families occurred in reference and intermediate sites in their study area (Dauvin & Ruellet, 2007). The results by Aguado-Giménez *et al*. (2015) contrast with the findings by Jahani *et al*. (2012) who used the BOPA index to detect adverse environmental conditions below fish cages and reported that abundance, biomass and diversity of invertebrates were low compared to reference conditions. Values of the polychaete/amphipod ratio are reliable only in strongly impacted areas (e.g. Dauvin, Andrade, De-la-Ossa-Carretero, Del-Pilar-Ruso, & Riera, 2016; Riera & De-la-Ossa-Carretero, 2014) since when the disturbance is mild, opportunistic taxa are not dominant and polychaete abundances do not reflect the disturbance level, while amphipod abundances may be effected by other variables, such as temperature (Wang *et al*., 2017). The findings by Aguado-Giménez *et al*. (2015) suggest that the use of the BOPA index may confound the interpretation of the EQS of the soft bottom habitat and use of this biotic index in conjunction with direct analysis of the macroinvertebrate assemblage is recommended.

Molluscs, which are an important benthic faunal group in terms of abundance, have been proposed by some workers (e.g. Charalampos & Drosos, 2008; Putro,

Muhammad, & Aininnur, 2017) to assess the effect of fish farming activities on macrobenthic assemblages. Mazzola and Sarà (2001) reported that bivalve molluscs present in the vicinity of fish farm cages contribute to assimilation of organic matter originating from the farms, thereby potentially helping to reduce excessive organic enrichment resulting from fish farming. However, the sensitivity of molluscs to nutrient enrichment seems to differ within the group, with some families responding with an increase in abundance and others with a decrease (e.g. Atalah & Crowe, 2012). The present results do not support the usefulness of molluscs as indicators of change resulting from fish farming activities: while Carditidae showed a low abundance over time at the impacted plot, no significant difference in the abundance of mollusc indicator taxa, number of taxa, diversity, and assemblage composition between impacted and control plots, was recorded over time, which may be attributed to the low similarity in mollusc family abundance recorded within the impacted and control plots.

The present study is unique in that it assesses the overall influence of tuna penning on benthic habitat over three years of tuna penning operations by using three impacted and six control locations with sampling sites nested within the locations. Such good spatial and temporal replication gives the possibility of detecting the effects of tuna penning on the seabed. However, the interpretation of statistical differences for most attributes of the macroinvertebrate assemblages under the influence of tuna penning over time was hindered due to significant interactions at the scale of locations; the pattern of response of a fish farm in one location could not be extrapolated to farms in other locations. Significant differences in the influence of tuna penning activities over time between different farm locations for the polychaete abundance, Shannon-Wiener diversity of amphipods, and assemblage composition of each of the four major faunal groups, prevented detection of statistically significant differences between the impacted and control plots, although the assemblage structure appeared different in terms of the polychaete/amphipod index. Furthermore, significant difference at the smaller scale of sites (a few meters) was indicated for several attributes of the macroinvertebrate assemblages, including number of families, Shannon-Wiener diversity and assemblage composition of polychaetes and amphipods; and polychaete/amphipod index.

The high spatio-temporal variability in attributes of the macrobenthic invertebrates in the vicinity of the three tuna farms recorded in the present study indicates that change in macroinvertebrate assemblages resulting from tuna penning activities differs between different farms. This observation corroborates the expectation that the level of influence of tuna penning activities on benthic habitat in the vicinity will vary with the fish stocking density, the length of time a farm has been in operation, and the feed management strategy adopted at the farm during the production period (e.g. Borja *et al*., 2009c). Given potential high variation in biological attributes at small spatial scales, the pattern of influence of a fish farm on benthic biota at one site cannot be extrapolated to other farms at different sites (e.g. Fernandez-Gonzalez *et al.*, 2013). Accordingly, inclusion of multiple impacted plots and good temporal replication in environmental monitoring studies of tuna farms is important.

Significant correlation at the control plots between attributes of the macroinvertebrate assemblages and sediment MSGS, POCC and PONC content 1-2 km away from the tuna cages, suggests that some influence of tuna penning may occur over a wider spatial extent than expected. A distance of 1 km between a fish farm and a control area would be expected to be sufficient for dispersal of farm wastes such that reference conditions are achieved (Porello *et al*., 2005); however the present results indicate otherwise. Significant spatio-temporal variation in the number of families and assemblage composition of polychaetes and amphipods amongst other biotic variables, was indicated between the two control plots at each farm site. It is also possible that other sources of organic enrichment apart from the tuna penning activities may be influencing the coastal waters where the tuna farms were located. While Malta is a small island with no rivers and generally shows oligotrophic conditions in coastal marine areas, the coastal waters found off the southern half of the island show higher nutrient loading compared to the northern half (Axiak, Pavlakis, Sieber, & Tarch, 2000), due to more intense coastal use (Mallia, Briguglio, Ellul, & Formosa, 2002). These observations highlight the importance of geographic variation in environmental impact monitoring studies, and emphasises the importance of including multiple reference areas in such studies.

Assessment of benthic biological indicators for evaluating the overall influence of tuna penning on soft bottom habitat must be interpreted with caution due to the recorded

high spatial and temporal variation. Attributes of the polychaete and amphipod assemblages appear to serve as good indicators of the environmental influence of tuna penning activities; lower (albeit not significantly) number and Shannon-Wiener diversity of polychaete and amphipod taxa was recorded at the impacted plots compared to the control plots over time, while the polychaete/amphipod index showed that the EQS at the impacted plots changed from 'Poor'/'Moderate' to 'Good' during the study period. Multivariate analyses indicated significantly higher dispersion of samples of the polychaete and amphipod assemblages over time at the impacted plots compared to the control plots, which is indicative of stressed macroinvertebrate assemblages. Of the polychaete families, the Capitellidae had an elevated abundance in the vicinity of the tuna cages, but no significant difference in abundance of polychaete indicator taxa was detected over time between impacted and control plots. On the other hand, abundance of indicator taxa, number of taxa, and Shannon-Wiener diversity of the mollusc assemblage showed no response. Hence, the use of mollusc taxa on their own as indicators of the environmental influence of tuna penning appears to be limited.

# CHAPTER 4

# DIFFERENCES IN MAGNITUDE AND SPATIAL EXTENT OF INFLUENCE OF TUNA PENNING ON BENTHIC MACROINVERTEBRATE ASSEMBLAGES

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## 4.1 Introduction

Farming of Atlantic bluefin tuna (*Thunnus thynnus thynnus* Linnaeus 1758) is a large sector of the aquaculture industry, which however has raised concerns on sustainability (see review by Metian *et al*., 2014). Atlantic Bluefin Tuna (ABT) is captured in the Mediterranean from the wild and transferred to cages for fattening (Camilleri, 2017) using whole bait fish as feed (Aguado *et al*., 2004; Vita & Marin, 2007). The uneaten feed-fish that accumulate below the tuna cages are the main source of pollution of the seabed (Aguado-Giménez *et al*., 2006; Mangion *et al*., 2014; Vita & Marin, 2007). The tuna are farmed at high stocking densities, which entail high feed input; however, these vary between different farms. As a result, one would expect differences in the magnitude and extent of adverse environmental impact, if present, between different farms. The potential adverse influence of tuna penning on the seabed may be reduced or eliminated when the cages are located in exposed sites that are characterised by deep waters, where strong sea currents prevail (Maldonado *et al*., 2005).

Several studies have addressed the environmental effects of tuna penning in the Mediterranean, including the potential adverse effects of ABT farming on nutrient levels in the water column and sediment (Aksu *et al*., 2010, 2016; Dal Zotto *et al*., 2016; Marin *et al*., 2007; Matijević *et al*., 2006, 2008; Vezzulli *et al*., 2008; Vita & Marin, 2007; Vita *et al*., 2004a), and water column microbial levels (Kapetanović *et al*., 2013). Other studies assessed the indirect effects of the ABT penning industry via the use of diesel fuel (Hospido & Tyedmers, 2005), the influence of ABT farming on *Posidonia oceanica* meadows (Kružić *et al*., 2014), the wild fish assemblages associated with the tuna pens (Šegvić Bubić *et al*., 2011), and the effect of ABT farming on trophic food-web linkages (Forrestal *et al*., 2012). Several studies on the influence of the activity on benthic macroinvertebrate assemblages in the vicinity of the tuna pens have also been published (Jahani *et al*., 2012; Mangion, Borg, Schembri, & Sanchez-Jerez, 2017; Mangion *et al*., 2014; Marin *et al*., 2007; Moraitis *et al*., 2013; Vezzulli *et al*., 2008; Vita & Marin, 2007). A comparison of the influence on the benthos between ABT farming and other Mediterranean fish farming activities, namely sea bass and sea bream rearing, is available in Sanz-Lázaro and Marin (2008).

Different conclusions have been reached on the magnitude and spatial extent of

adverse effects of fish farming on the seabed because the experimental design, research method and indicators used, as well as local environmental factors, vary widely between different study sites (Kalantzi & Karakassis, 2006). To properly address the environmental impact of ABT farming on benthic habitat, it is desirable to include multiple spatial scales in the sampling design (Wiens, 1989). Determination of appropriate spatial scales at which potential environmental impacts of aquaculture may be investigated is necessary to enable proper assessment of patterns of variation in the influence of the activity on the marine environment (Fernandez-Gonzalez *et al*., 2013). Several studies have assessed patterns of variation in the influence of fish farming on benthic habitat at a number of spatial scales (e.g. Gyllenhammar & Håkanson, 2005; Fernandez-Gonzalez *et al*., 2013), but in the case of tuna penning this aspect has not been given sufficient attention (but see Moraitis *et al*., 2013; Vita & Marin, 2007).

The use of polychaetes (e.g. Aguado-Giménez *et al*., 2015; Martinez-Garcia *et al*., 2013; Sutherland *et al*., 2007; Tomassetti & Porrello, 2005) and amphipods (e.g. Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011) as biological indicators of fish farming influence on benthic habitat is well known. The polychaete/amphipod (BOPA) ratio is a benthic index developed for the European Water Framework Directive (WFD, 2000/60/EC) (Dauvin & Ruellet, 2007; Gomez-Gesteira & Dauvin, 2000), that has been previously used to classify coastal waters under the influence of fish farming activities (e.g. Aguado-Giménez *et al*., 2015; Jahani *et al*., 2012; Mangion *et al*., 2017) into 'High', 'Good', 'Moderate', 'Poor' or 'Bad' Ecological Quality Status (EQS) classes (Dauvin & Ruellet, 2007; Gomez-Gesteira & Dauvin, 2000). The BOPA index uses frequency data and the proportion of organisms in each category, which render it independent of sampling protocols that utilize different mesh sizes and measurements used to express the abundance of organisms per unit area. Another major advantage of the BOPA index is the reduced taxonomic effort required to assess the Ecological Quality Status (EQS) of the marine environment. The BOPA index has been applied to measure the impact of various environmental disturbances, proving effective in distinguishing the presence of hydrocarbons (Dauvin & Ruellet, 2007; Gomez-Gesteira & Dauvin, 2000) and sewage discharges (De-la-Ossa-Carretero *et al*., 2009) at certain sites, such as oyster culture areas (Bouchet & Sauriau, 2008) and harbors (Ingole, Sivadas, Nanajkar, Sautya, & Nag, 2009). However, the BOPA index tends to overestimate the EQS compared with

other benthic indices (see De-la-Ossa-Carretero & Dauvin, 2010). A modification of the BOPA index has been proposed by Aguado-Giménez *et al*. (2015) to improve its performance at Mediterranean sites affected by fish farming activities.

The main aim of the present study was to assess the magnitude and spatial extent of the impact of tuna penning on soft bottom polychaete and amphipod assemblages using a hierarchical spatial design that incorporates different spatial levels; from tens of meters to a few kilometers. The considered attributes are: abundance of three selected indicator taxa, total number of taxa, Shannon-Wiener diversity, assemblage composition of polychaete and amphipod taxa, and the polychaete/amphipod index (as defined by Aguado-Giménez *et al*., 2015). In the present study, the hypothesis that particulate organic matter originating from tuna cages and settling on the seabed leads to changes in the invertebrate assemblages associated with the soft bottom habitat, was tested. To achieve this, data on sediment physico-chemical attributes, namely: w/w feed-fish bone content (FFBC), which represents the uneaten feed-fish that decomposed on the seabed; mean sediment grain size (MSGS), percent organic carbon content (POCC), and percent organic nitrogen content (PONC), was used. Three tuna farms located in the Maltese Islands and differing in size and feed management regime, were used in the present assessment.

## 4.2 Material and methods

### 4.2.1 Study sites and sampling

The three Maltese tuna farms considered in the present study were located 1 km offshore (Figure 4.1) where the seabed consisted of soft sediment. One farm was located off the northeastern coast, where the water depth was some 45 m – 50 m, while the other two farms were located some 1.5 km apart off the southeastern coast, where the water depth was some 42 m – 53 m. The northeastern farm (NEF) had eight tuna cages with a maximum total annual capacity of 2500 t, while the two southeastern farms were smaller (maximum total annual capacity of 1500 t each); one having three cages (southeastern ‘Farm 1’ [SEF 1]) and the other (southeastern ‘Farm 2’ [SEF 2]) having four cages (International Commission for the Conservation of Atlantic Tunas

[ICCAT], 2011). All three farms utilized cages having a diameter of some 50 m and a height of around 25 m. The tuna stocking density was circa 100 ± 200 t per cage, and the fish were fed the equivalent of 3-4 % of the fish biomass per day, divided over two feeding sessions (tuna farm managers, personal communication). The feed consisted of whole bait fish; namely mackerel, sardines, squid and prawn; and the ratio of food (based on the wet weight of the feed) that is converted into tuna biomass is around 10-15:1 (tuna farm managers, personal communication). However, the feeding regime is expected to differ between the different farms as a result of adaptive management to natural environmental factors (e.g. sea current strength) and varying growth rate of the tuna.

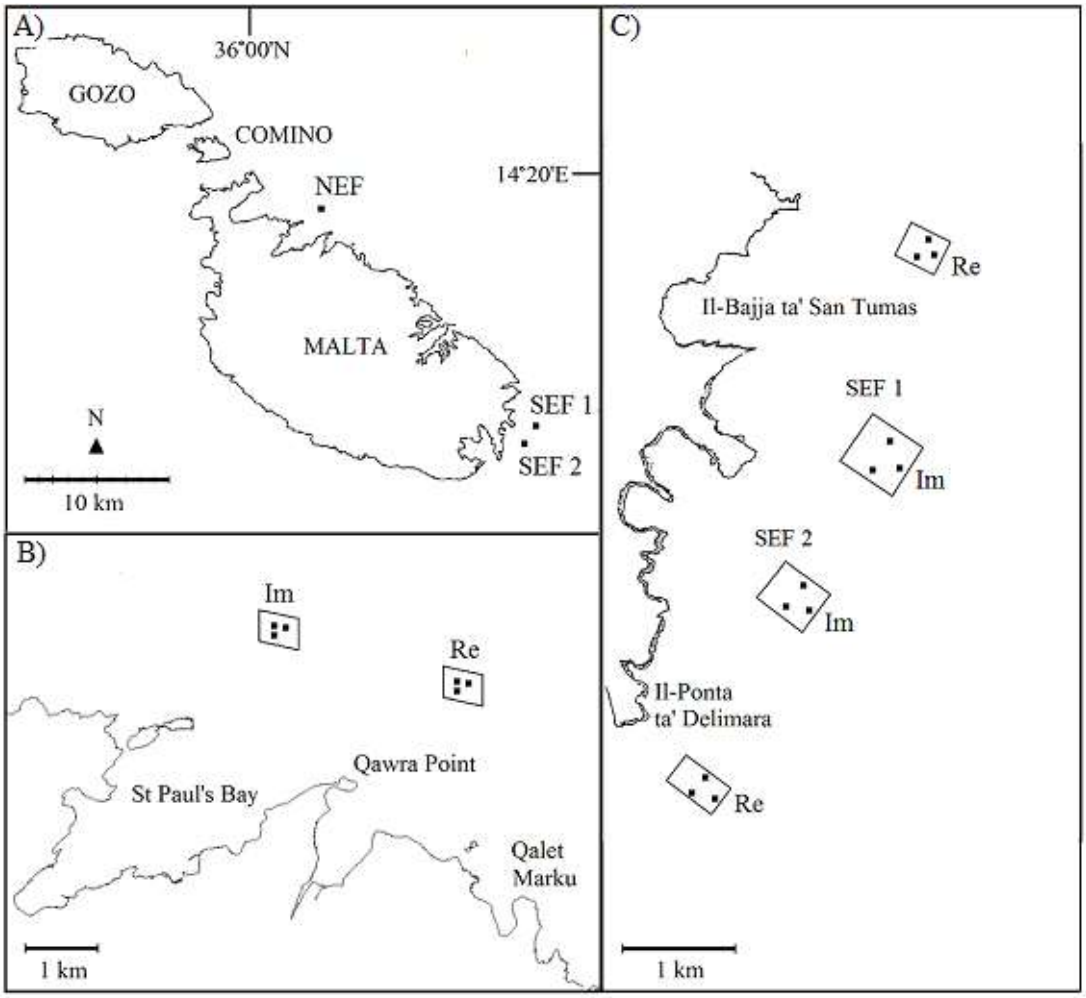


**Figure 4.1** Map of the Maltese Islands showing: (a) the locations of the three tuna farms; (b) the northeastern farm; and (c) the two southeastern farms; from where samples for sediment quality and benthic macrofaunal studies were collected. Im = impacted plot, Re = reference plot, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2'

The sampling design incorporated three fixed, orthogonal factors: (a) Before/After (BA), with two sampling periods: (i) November 2000 at the NEF, October 2002 at the SEF 1, and June 2001 at the SEF 2 'before' initiation of the tuna penning activities; and (ii) November 2001 at the NEF, October 2003 at the SEF 1, and June 2002 at the SEF 2, 'after' initiation of the activity; (b) Location (Lo), with three farms: (i) the NEF, (ii) SEF 1, and (iii) SEF 2; and (c) Plot (Pl), measuring some 300 m by 500 m, with two treatments: (I) 'impacted' plot, i.e. the seabed area where the tuna cages were sited; and (ii) 'reference' plot, located some 1 km – 1.5 km away from the cages. A random factor 'Site' (Si) was nested within the 'BA x Lo x Pl' interaction, with sites separated at the scale of hundreds of meters. Three sampling sites were allotted to each level of the three-way interaction, since the minimum number of cages at any one of the farms was three, such that a total of 18 sampling sites are included in the sampling design. The latitude/longitude coordinates and depth of the 18 sampling sites are given in Table 4.1.

**Table 4.1** Latitude/longitude coordinates and depth of the reference sites shown in Figure 4.1. The impacted plot at the NEF was centered on N35º 58.66'/E14º 25.16'; at the SEF 1 on N35º 50.17'/E14º 35.11'; and at the SEF 2 on N35º 49.85'/E14º 34.67'; while samples were also collected from the seabed area directly below the cages.

| Farm | Site | Latitude/Longitude | Depth (m) |
|---|---|---|---|
| NEF | S1 | N35º 58.32/E14º 26.72 | 50 |
| | S2 | N35º 58.18/E14º 26.85 | 50 |
| | S3 | N35º 58.12/E14º 26.72 | 48 |
| SEF 1 | S1 | N35º 51.58/E14º 35.42 | 47 |
| | S2 | N35º 51.50/E14º 35.48 | 47 |
| | S3 | N35º 51.49/E14º 35.37 | 45 |
| SEF 2 | S1 | N35º 49.02/E14º 34.15 | 48 |
| | S2 | N35º 48.83/E14º 34.22 | 49 |
| | S3 | N35º 48.87/E14º 34.00 | 48 |

Sampling was carried out using a 0.1 $m^2$ van Veen grab. Three replicate grab samples for benthic macrofaunal studies and one grab sample for sediment physico-chemical studies were collected at each of the eighteen sampling sites. The collected samples were live-sieved (0.5 mm mesh) on board the vessel and afterward temporarily preserved in 10% formalin.

In the laboratory, samples for faunal studies were sorted for polychaetes and amphipods after washing on a 0.5 mm mesh. Specimens were identified to family level

(see Karakassis & Hatziyanni, 2000; Olsgard & Somerfield, 2000) and enumerated to obtain estimates of number of families and abundance per grab sample. For sediment physico-chemical studies, sub-samples were frozen at -20°C for later analysis to determine the POCC, PONC and FFBC, while another sub-sample was oven dried for granulometric analysis. Analysis of the sediment to determine the FFBC was carried out by sorting fish bones from the sediment using forceps under a dissecting microscope. POCC in the sediment was determined by wet oxidation using a chromic acid-sulfuric acid mixture and titration of the evolved carbon dioxide (see Walkley & Black, 1934). PONC in the sediment was determined by the Kjeldhal method, i.e. by digestion in concentrated sulfuric acid containing a copper sulfate catalyst, addition of excess strong alkali, and condensation of the ammonia given off for titration. Measurement of MSGS was carried out according to Buchanan (1984) (see Holme & McIntyre, 1984).

Unpublished data on sea current direction and velocity, at water depths ranging from 1 to 10 m, were available from surveys undertaken once every 3 months at the northeastern and southeastern farm sites, during the period 2010-2017, using the Lagrange method (see Bennett, 2006).

### 4.2.2 Data analyses

Indicator taxa at family level were selected as the three most abundant (in terms of number of individuals) macroinvertebrates (see Morrisey, 1992) before tuna penning activities were initiated. The polychaete/amphipod (BOPA-Fish farming [BOPA-FF]) index was calculated using BOPA = log (($f_P$ / $f_A$ +1) +1 ); where '$f_P$' is the frequency of polychaetes tolerant to organic enrichment resulting from fish farming activities, as identified by Martinez-Garcia *et al*. (2013) (see Aguado-Giménez *et al*., 2015), and '$f_A$' is the frequency of amphipod individuals excluding the genus *Jassa* (Dauvin & Ruellet, 2007). Boundary values between 'High' ($0.00 \leq x \leq 0.05$), 'Good' ($0.05 < x \leq 0.14$), 'Moderate' ($0.14 < x \leq 0.19$), 'Poor' ($0.19 < x \leq 0.27$), and 'Bad' ($0.27 < x \leq 0.30$) EQS classes are as given in Dauvin and Ruellet (2007).

Four-factor univariate permutational analysis of variance (PERMANOVA) (Anderson, 2001) was carried out using a Euclidean similarity matrix to test the hypothesis of no

difference in tuna penning activities between different farms in terms of: (i) abundance of selected indicator polychaete families Maldanidae, Paraonidae and Glyceridae, and of amphipod families Lysianassidae, Phoxocephalidae and Urothoidae; (ii) number and Shannon-Wiener diversity of polychaete and amphipod families; and (iii) polychaete/amphipod (BOPA-FF) index as defined by Aguado-Giménez *et al*. (2015). Separate univariate PERMANOVA was carried out using a Euclidean similarity matrix to test the hypothesis of no difference in tuna penning activities between different farms in terms of the sediment MSGS, POCC and PONC, using a model with three fixed, orthogonal factors 'BA', 'Lo' and 'Pl', and treating the levels of 'Si' as replicates. When the PERMANOVA indicated a significant difference, the source of significant difference was identified for the highest interaction term using *a posteriori* pair-wise tests.

Four-factor multivariate PERMANOVA (Anderson, 2001; McArdle & Anderson, 2001) was used on a Bray Curtis similarity matrix calculated from fourth-root transformed (Clarke & Warwick, 2001) family abundance data to test the hypothesis of no difference in tuna penning activities between different farms in terms of the assemblage composition of polychaetes and amphipods. A permutational multivariate dispersion test (PERMDISP) (Anderson, 2004, 2006) was then used to calculate differences in within-group dispersion using the sample distance to the centroid of each of the factors. In both PERMANOVA and PERMDISP tests, a total of 9999 unrestricted permutations of raw data were used, with $\alpha$ set at 0.05. Principal coordinate analysis (PCO) (Anderson, 2003) was run and the results were plotted to show differences in tuna penning influence on polychaete and amphipod assemblage composition between the different farms. The three-factor model was used to test for significant differences in sediment physico-chemical attributes by carrying out a similar multivariate analysis using a D1 Euclidean similarity matrix calculated from environmental data that was normalised to homogenize the different units (Clarke & Warwick, 2001). The three most important taxa contributing to dissimilarity in polychaete and amphipod assemblages at the impacted and reference plots from before to after tuna penning activities, and between the impacted and reference plots before and after initiation of the tuna penning activities, were identified for each farm using the similarity percentages of species contributions (SIMPER) method (Clarke & Warwick, 2001), and *a posteriori* univariate PERMANOVA was run (with $\alpha$ set at

0.05) on abundance values of taxa that contributed most to the dissimilarity of samples at each farm using 'Si(BA x Pl)'. To determine which sediment physico-chemical variable, or combination of variables, best explained the observed variation in the macroinvertebrate assemblages, the BEST routine for matching biotic data with environmental attributes (Clarke & Gorley, 2006) was carried out using the Spearman rank correlation method and D1 Euclidean similarity measure, at the level of the two-way interaction terms, since the number of replicates at the level of 'BA x Lo x Pl' was too low. All the analyses were undertaken using PRIMER v.7.0.11 (PRIMER software; Clarke & Gorley, 2006) and the PERMANOVA+ v.1.0 add-on package (Anderson *et al*., 2008).

## 4.3 Results

### 4.3.1 Univariate data analyses

#### 4.3.1(i) Macroinvertebrate assemblages

A total of 5,750 individuals from 26 polychaete families, and 2,103 individuals from 22 amphipod families, were collected. The top families (in terms of number of individuals) that characterised the polychaete and amphipod assemblages at the three tuna farms before farming activities commenced were: Maldanidae, Paraonidae and Glyceridae (polychaetes), and Lysianassidae, Phoxocephalidae and Urothoidae (amphipods) (Figure 4.2).

PERMANOVA indicated no significant difference in the interaction term 'BA x Lo x Pl' for abundance of polychaetes and amphipods, while 'BA x Lo' was significant for abundance of Glyceridae ($p < 0.05$) and Urothoidae ($p < 0.01$), 'BA x Pl' was significant for abundance of Urothoidae ($p < 0.001$), and 'Pl x Lo' was significant for abundance of Maldanidae ($p < 0.05$), Glyceridae ($p < 0.01$), Urothoidae ($p < 0.001$) and Phoxocephalidae ($p < 0.01$) (Table 4.2). Pair-wise tests showed that the abundance of Glyceridae recorded from the NEF impacted/reference plots increased significantly ($p < 0.05$) following the tuna penning activities, while the abundance of Urothoidae ($p < 0.01$) and Phoxocephalidae ($p < 0.001$) was significantly lower at the NEF impacted

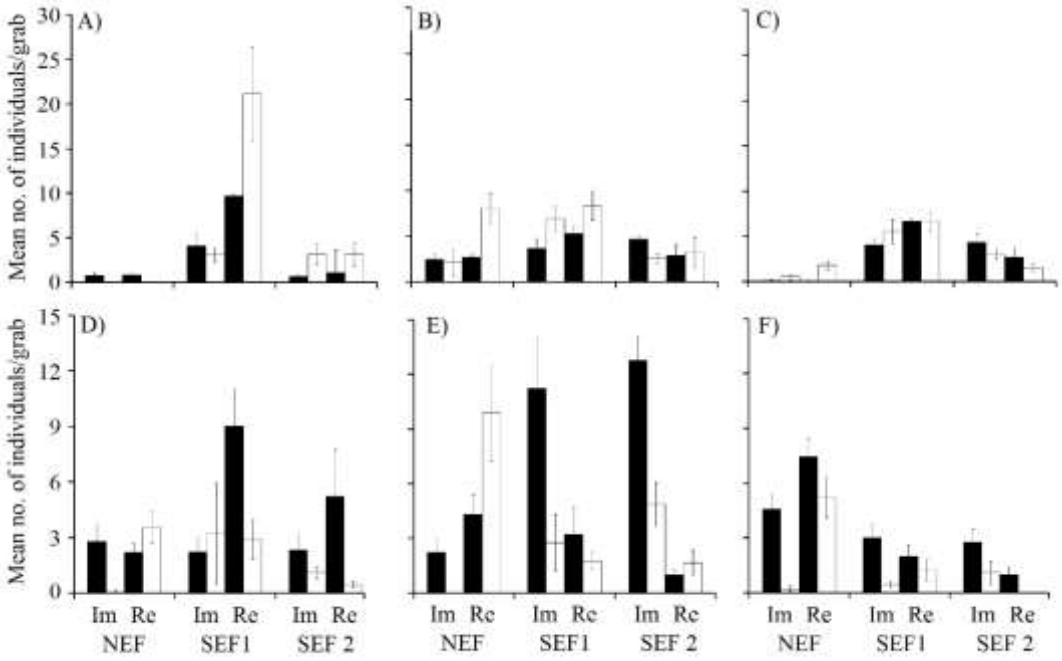


**Figure 4.2** Mean values (± SE) per grab of: number of individuals of polychaete indicator taxa (a) Maldanidae, (b) Paraonidae, (c) Glyceridae, and amphipod indicator taxa (d) Lysianassidae, (e) Urothoidae, (f) Phoxocephalidae, recorded before (black bars) and after (white bars) tuna-penning activities at the impacted (Im) and reference (Re) plots of the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1) and southeastern 'Farm 2' (SEF 2).

plot compared with the NEF reference plot before/after the tuna penning activities (Figure 4.2, Table 4.2). At the southeastern farms, the abundance of Urothoidae recorded from the impacted/reference plots decreased significantly ($p_{SEF\,1} < 0.05$, $p_{SEF\,2} < 0.001$) following the tuna penning activities. The abundance of Phoxocephalidae was significantly higher ($p < 0.05$) at the SEF 1 impacted plot compared with the SEF 1 reference plot, while the abundance of Glyceridae ($p < 0.05$), Urothoidae ($p < 0.05$) and Phoxocephalidae ($p < 0.001$) was significantly higher at the SEF 2 impacted plot compared with the SEF 2 reference plot, before/after the tuna penning activities (Figure 4.2, Table 4.2).

PERMANOVA indicated a significant difference in the abundance of Lysianassidae for 'BA' ($p < 0.05$), and in the abundance of Paraonidae for 'Lo' ($p < 0.05$) (Table 4.2). Pair-wise tests showed that the overall abundance of Lysianassidae decreased significantly ($p < 0.05$) following initiation of tuna penning, while the overall abundance of Paraonidae was significantly higher ($p < 0.05$) at the SEF 1 compared with the NEF and SEF 2 (Figure 4.2, Table 4.1). PERMANOVA also indicated a

**Table 4.2** Results of four-factor, univariate PERMANOVA for number of individuals of selected indicator taxa, number of families and Shannon-Wiener diversity of polychaetes and amphipods, and polychaete/amphipod ratio, with *a posteriori* pair-wise comparisons for the significant factors and interaction terms. Level of significance set at 0.05. Df = Degrees of freedom, NI = number of individuals; Polychaete indicator taxon 1 = Maldanidae, 2 = Paraonidae, 3 = Glyceridae (3); Amphipod indicator taxon 1 = Lysianassidae, 2 = Urothoidae, 3 = Phoxocephalidae; NFa = Number of families, ShW = Shannon-Wiener diversity, BOPA-FF = BOPA-Fish farming family index, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', Im = impacted plot, Re = reference plot, Be = Before, Af = After, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | | | | Polychaetes | | | | | | Amphipods | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | | | df | NI 1 | NI 2 | NI 3 | NFa | ShW | NI 1 | NI 2 | NI 3 | NFa | ShW |
| Before/After = BA | | | 1 | ns | ns | ns | * | *** | * | ** | *** | *** | *** |
| Location = Lo | | | 2 | *** | * | *** | *** | *** | ns | ns | *** | *** | *** |
| Plot = Pl | | | 1 | * | ns | ns | ** | *** | ns | ** | ns | *** | *** |
| BA x Lo | | | 2 | ns | ns | * | ns | ** | ns | ** | ns | *** | *** |
| BA x Pl | | | 1 | ns | ns | ns | ns | * | ns | *** | ns | ns | ns |
| Pl x Lo | | | 2 | * | ns | ** | ns | *** | ns | *** | ** | *** | *** |
| BA x Lo x Pl | | | 2 | ns | ns | ns | ns | ** | ns | ns | ns | * | *** |
| Site = Si (BA x Lo x Pl) | | | 24 | *** | * | ns | * | * | * | ** | ns | ns | ns |
| Residual | | | 72 | | | | | | | | | | |
| Total | | | 107 | | | | | | | | | | |
| *Pair-wise tests -: 'BA x Lo x Pl'* | | | | | | | | | | | | | |
| NEF | Im | | | - | - | - | - | > *** | - | - | - | > ** | > ** |
| | Re | | | - | - | - | - | ns | - | - | - | ns | ns |
| SEF 1 | Im | Be, Af | | - | - | - | - | ns | - | - | - | ns | ns |
| | Re | | | - | - | - | - | ns | - | - | - | ns | ns |
| SEF 2 | Im | | | - | - | - | - | ns | - | - | - | > * | ns |
| | Re | | | - | - | - | - | ns | - | - | - | > ** | > ** |
| Be | NEF | | | - | - | - | - | < * | - | - | - | < ** | < * |
| | SEF 1 | | | - | - | - | - | ns | - | - | - | ns | ns |
| | SEF 2 | Im, Re | | - | - | - | - | ns | - | - | - | ns | ns |
| Af | NEF | | | - | - | - | - | < *** | - | - | - | < ** | < *** |
| | SEF 1 | | | - | - | - | - | ns | - | - | - | ns | < * |

**Table 4.2** Continued

| Source of Variation | | | Polychaetes NI 1 | NI 2 | NI 3 | NFa | ShW | Amphipods NI 1 | NI 2 | NI 3 | NFa | ShW |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Af | SEF 2 | | - | - | - | - | ns | - | - | - | ns | ns |
| Be | Im | NEF, SEF 1 | - | - | - | - | ns | - | - | - | < * | ns |
| | | NEF, SEF 2 | - | - | - | - | < ** | - | - | - | < ** | < ** |
| | | SEF 1, SEF 2 | - | - | - | - | ns | - | - | - | < * | < ** |
| | Re | NEF, SEF 1 | - | - | - | - | ns | - | - | - | ns | ns |
| | | NEF, SEF 2 | - | - | - | - | ns | - | - | - | ns | < ** |
| | | SEF 1, SEF 2 | - | - | - | - | ns | - | - | - | ns | < * |
| Af | Im | NEF, SEF 1 | - | - | - | - | < ** | - | - | - | < ** | < ** |
| | | NEF, SEF 2 | - | - | - | - | < *** | - | - | - | < * | < ** |
| | | SEF 1, SEF 2 | - | - | - | - | ns | - | - | - | ns | ns |
| | Re | NEF, SEF 1 | - | - | - | - | ns | - | - | - | ns | ns |
| | | NEF, SEF 2 | - | - | - | - | ns | - | - | - | > * | > * |
| | | SEF 1, SEF 2 | - | - | - | - | ns | - | - | - | > ** | > * |
| *Pair-wise tests -: 'BA x Lo', 'BA x Pl', and 'Pl x Lo'* | | | | | | | | | | | | |
| NEF1 | | | - | - | < * | - | - | - | - | ns | - | - |
| SEF 1 | Be, Af | | - | - | ns | - | - | - | - | > * | - | - |
| SEF 2 | | | - | - | ns | - | - | - | - | > *** | - | - |
| Be | NEF, SEF 1 | | - | - | < *** | - | - | - | - | ns | - | - |
| | NEF, SEF 2 | | - | - | < *** | - | - | - | - | > *** | - | - |
| | SEF 1, SEF 2 | | - | - | ns | - | - | - | - | ns | - | - |
| Af | NEF, SEF 1 | | - | - | < *** | - | - | - | - | > * | - | - - |
| | NEF, SEF 2 | | - | - | ns | - | - | - | - | ns | - | - - |
| | SEF 1, SEF 2 | | - | - | > *** | - | - | - | - | ns | - | - - |
| Im | Be, Af | | - | - | - | - | - | - | - | > *** | - | - - |
| Re | | | - | - | - | - | - | - | - | ns | - | - - |
| Be | Im, Re | | - | - | - | - | - | - | - | > *** | - | - - |
| Af | | | - | - | - | - | - | - | - | < * | - | - - |

**Table 4.2** Continued

| | | Polychaetes | | | | | Amphipods | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | | NI 1 | NI 2 | NI 3 | NFa | ShW | NI 1 | NI 2 | NI 3 | NFa | ShW |
| *Pair-wise tests -: 'BA x Lo', 'BA x Pl', and 'Pl x Lo'* | | | | | | | | | | | |
| Im | NEF, SEF 1 | < ** | - | < *** | - | - | - | ns | > ** | - | - |
| | NEF, SEF 2 | ns | - | < *** | - | - | - | ns | > *** | - | - |
| | SEF 1, SEF 2 | ns | - | ns | - | - | - | ns | ns | - | - |
| Re | NEF, SEF 1 | < * | - | < *** | - | - | - | > ** | > ** | - | - |
| | NEF, SEF 2 | ns | - | < * | - | - | - | > *** | > *** | - | - |
| | SEF 1, SEF 2 | > * | - | > *** | - | - | - | ns | ns | - | - |
| NEF | | ns | - | ns | - | - | - | < ** | < *** | - | - |
| SEF 1 | Im, Re | ns | - | ns | - | - | - | ns | > * | - | - |
| SEF 2 | | ns | - | > * | - | - | - | > * | > *** | - | - |
| *Pair-wise tests -: 'BA', 'Lo', and 'Pl'* | | | | | | | | | | | |
| | Be, Af | - | - | - | > * | - | > * | - | - | - | - |
| | NEF, SEF 1 | - | < * | - | < *** | - | - | - | - | - | - |
| | NEF, SEF 2 | - | ns | - | < *** | - | - | - | - | - | - |
| | SEF 1, SEF 2 | - | > * | - | ns | - | - | - | - | - | - |
| | Im, Re | - | - | - | < ** | - | - | - | - | - | - |

significant difference in ‘Si(BA x Lo x Pl)’ for abundance of Maldanidae ($p < 0.001$), Paraonidae ($p < 0.05$), Lysianassidae ($p < 0.05$) and Urothoidae ($p < 0.01$) (Table 4.2).

PERMANOVA indicated a significant difference in Shannon-Wiener diversity of polychaetes ($p < 0.01$), number of amphipod families ($p < 0.05$), and Shannon-Wiener diversity of amphipods ($p < 0.001$) for the interaction term ‘BA x Lo x Pl’ (Table 4.2). Pair-wise tests showed that following initiation of the tuna penning activities, Shannon-Wiener diversity of polychaetes ($p < 0.001$), number of amphipod families ($p < 0.01$), and Shannon-Wiener diversity of amphipods ($p < 0.01$) decreased significantly at the NEF impacted plot (Figure 4.3, Table 4.2). The number of amphipod families at the SEF 2 impacted ($p < 0.05$) and reference plots ($p < 0.01$), and Shannon-Wiener diversity of amphipods at the SEF 2 reference plot ($p < 0.05$), decreased significantly in the same period (Figure 4.3, Table 4.2).

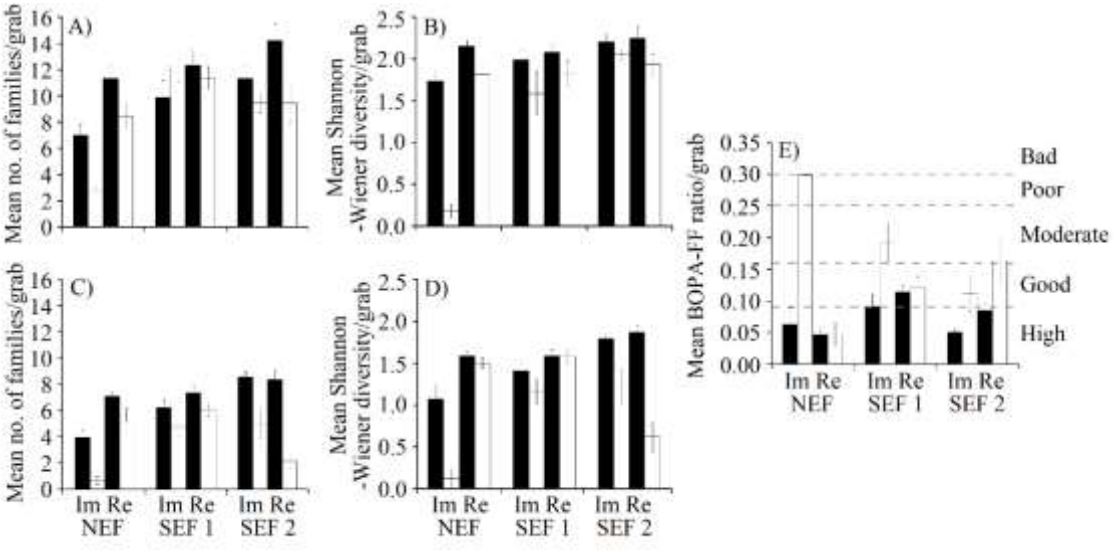


**Figure 4.3** Mean values (± SE) per grab of: (a, c) total number of families and (b, d) Shannon-Wiener diversity of (a, b) polychaetes and (c, d) amphipods, and (e) of the polychaete/amphipod ratio recorded before (black bars) and after (white bars) tuna-penning activities at the impacted (Im) and reference (Re) plots of the northeastern farm (NEF), southeastern ‘Farm 1’ (SEF 1), and southeastern ‘Farm 2’ (SEF 2).

PERMANOVA indicated significant differences in the number of polychaete families for ‘BA’ ($p < 0.05$), ‘Lo’ ($p < 0.001$), and ‘Pl’ ($p < 0.01$) (Table 4.2). Pair-wise tests showed that the overall number of polychaete families decreased significantly ($p < 0.05$) following tuna penning activities, and was significantly low ($p < 0.001$) at the

NEF compared with the SEF 1 and the SEF 2 and at the impacted plots compared with the reference plots ($p < 0.01$) (Figure 4.3, Table 4.2). PERMANOVA also indicated a significant difference ($p < 0.05$) in 'Si(BA x Lo x Pl)' for the number of families and Shannon-Wiener diversity of polychaetes (Table 4.2).

PERMANOVA indicated a significant difference in BOPA-FF for the interaction term 'BA x Lo x Pl' ($p < 0.01$) (Table 4.2). Values of the mean BOPA-FF index indicated 'Good'/'High' EQS at the NEF and the SEF 2, and 'Good' EQS at the SEF 1, at the impacted and reference plots prior to the initiation of tuna penning activities, while pair-wise tests indicated no significant difference in the BOPA-FF index for that period (Figure 4.3, Table 4.2). Following the initiation of tuna penning activities, BOPA-FF increased significantly at the NEF ($p < 0.01$) and the SEF 2 ($p < 0.05$) impacted plots. The mean EQS was 'Bad' at the NEF impacted plot and 'High' at the NEF reference plot; pair-wise tests showed that BOPA-FF was significantly high ($p < 0.001$) at the NEF impacted plot compared with the NEF reference plot in the same period. There was no significant difference in the BOPA-FF index between mean values of 'Moderate' and 'Good' EQS, respectively, at the SEF 1 impacted and reference plots following the tuna penning activities, nor between mean values of 'Good' and 'Moderate' EQS, respectively, at the SEF 2 impacted and reference plots in the same period. Pair-wise tests showed that the BOPA-FF was significantly high ($p_{SEF\,1} < 0.05$, $p_{SEF\,2} < 0.001$) at the NEF impacted plot compared with the impacted plots of the two southeastern farms following the farming activities. No significant difference in BOPA-FF was detected between the impacted plots of the two southeastern farms in the same period (Figure 4.3, Table 4.2).

### 4.3.1(ii) Sediment physico-chemical attributes

The sediment FFBC recorded below fish cages following the tuna penning activities was higher at the NEF (2.33% $\pm$ 3.12%) compared with the two southeastern farms, and higher at the SEF 1 (1.59% $\pm$ 2.66%) compared with the SEF 2 (0.04% $\pm$ 0.06%).

PERMANOVA indicated significant differences in sediment POCC and PONC for 'BA x Lo x Pl' ($p < 0.05$) (Table 4.3). Pair-wise tests showed that, following initiation of tuna penning, POCC increased significantly ($p < 0.05$) at the NEF impacted plot (Figure 4.4, Table 4.3). The general increase in POCC recorded at the SEF 1 impacted plot, and at the SEF 2 reference plot, was not significant. PONC increased significantly ($p < 0.05$) at the SEF 1 reference plot following the tuna penning activities, and was significantly high ($p < 0.05$) at the SEF 1 reference plot compared with the NEF reference plot in the same period (Figure 4.4, Table 4.3). A general increase in PONC following tuna penning activities was observed at the NEF impacted plot, while no significant difference was detected in this sediment attribute at the NEF from before to after initiation of the tuna penning activities, and between the impacted and reference plot afterwards (Figure 4.4, Table 4.3). The general trend in MSGS was similar before and after the tuna penning activities, at the impacted and reference plots of each of the three tuna farms, with no significant differences indicated for 'BA', 'Lo', and 'Pl'; and the interactions terms (Figure 4.4, Table 4.3).

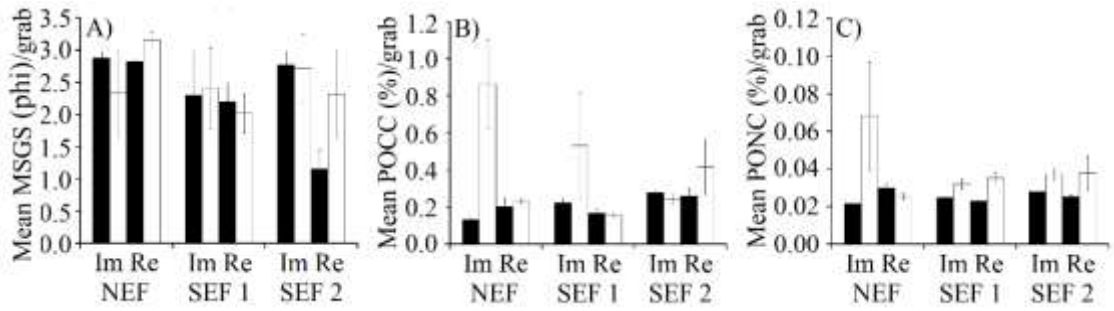


**Figure 4.4** Mean values (± SE) per grab of: (a) sediment mean grain size (MSGS), (b) percent organic carbon content (POCC) in sediment and (c) percent organic nitrogen content (PONC) in sediment recorded before (black bars) and after (white bars) tuna penning activities at the impacted (Im) and reference (Re) plots of the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1), and southeastern 'Farm 2' (SEF 2).

### 4.3.1(iii) Relationship between sediment attributes and macroinvertebrates

BEST analysis showed that a combination of MSGS and POCC was significantly correlated with the Shannon-Wiener diversity of polychaetes recorded overall during the study period at the NEF impacted plot ($\rho = 0.607$, $p < 0.05$), and with the Shannon-Wiener diversity of polychaetes ($\rho = 0.668$, $p < 0.05$), number of amphipod families ($\rho = 0.613$, $p < 0.05$), and Shannon-Wiener diversity of amphipods ($\rho = 0.810$, $p <$

**Table 4.3** Results of three-factor, univariate PERMANOVA calculated from a Euclidean similarity matrix of normalised sediment physico-chemical data, with *a posteriori* pair-wise comparisons for the significant third-order interaction term. Level of significance set at 0.05. Df = Degrees of freedom, MSGS = mean sediment grain size (phi), POCC = percent organic carbon content, PONC = percent organic nitrogen content, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', Im = impacted plot, Re = reference plot, Be = before, Af = after

| Source of Variation | | | df | MSGS | POCC | PONC |
|---|---|---|---|---|---|---|
| Before/After = BA | | | 1 | ns | ** | ** |
| Location = Lo | | | 2 | ns | * | ns |
| Plot = Pl | | | 1 | ns | ns | ns |
| BA x Pl | | | 2 | ns | ns | ns |
| BA x Lo | | | 1 | ns | ns | ns |
| Pl x Lo | | | 2 | ns | ns | ns |
| BA x Lo x Pl | | | 2 | ns | * | * |
| Residual | | | 24 | | | |
| Total | | | 35 | | | |
| *Pair-wise tests -: 'BA x Lo x Pl'* | | | | | | |
| NEF | Im | Be, Af | | - | < * | ns |
| | Re | | | - | ns | ns |
| SEF 1 | Im | | | - | ns | ns |
| | Re | | | - | ns | < * |
| SEF 2 | Im | | | - | ns | ns |
| | Re | | | - | ns | ns |
| Be | NEF | Im, Re | | - | ns | < * |
| | SEF 1 | | | - | ns | ns |
| | SEF 2 | | | - | ns | ns |
| Af | NEF | | | - | ns | ns |
| | SEF 1 | | | - | ns | ns |
| | SEF 2 | | | - | ns | ns |
| Be | Im | NEF, SEF 1 | | - | < * | ns |
| | | NEF, SEF 2 | | - | < ** | < * |
| | | SEF 1, SEF 2 | | - | ns | > ** |
| | Re | NEF, SEF 1 | | - | ns | > ** |
| | | NEF, SEF 2 | | - | ns | ns |
| | | SEF 1, SEF 2 | | - | ns | ns |
| Af | Im | NEF, SEF 1 | | - | ns | < * |
| | | NEF, SEF 2 | | - | ns | ns |
| | | SEF 1, SEF 2 | | - | ns | ns |
| | Re | NEF, SEF 1 | | - | > ** | < * |
| | | NEF, SEF 2 | | - | ns | ns |
| | | SEF 1, SEF 2 | | - | ns | ns |

0.01), recorded overall at the NEF impacted and reference plots after the tuna penning activites (Table 4.4).

At the SEF 1, BEST analysis showed significant correlation between the POCC and number of polychaete families ($\rho = 0.852$, $p < 0.05$), and between a combination of POCC and PONC, and Shannon-Wiener diversity of polychaetes ($\rho = 0.921$, $p < 0.001$), recorded from the impacted plot before/after the tuna penning activities (Table 4.4). BEST analysis also showed a significant correlation: (i) between the POCC and abundance of Lysianassidae ($\rho = 0.815$, $p < 0.05$) and the Shannon-Wiener diversity of amphipods ($\rho = 0.871$, $p < 0.01$) at the SEF 1 impacted/reference plots after the tuna penning activities; (ii) between a combination of MSGS and POCC and the abundance of Glyceridae ($\rho = 0.604$, $p < 0.05$) and the BOPA-FF index ($\rho = 0.754$, $p < 0.05$) recorded from the SEF 1 reference plot before/after the tuna penning activities; and (iii) between the PONC and abundance of Glyceridae ($\rho = 0.931$, $p < 0.01$) recorded overall from the SEF 1 impacted/reference plots before initiation of the tuna penning activities (Table 4.4).

At the SEF 2, BEST analysis showed a significant correlation: (i) between PONC and abundance of Paraonidae ($\rho = 0.671$, $p < 0.05$) recorded overall from the impacted plot before/after the tuna penning activities; (ii) between a combination of MSGS and POCC and abundance of Lysianassidae ($\rho = 0.865$, $p < 0.05$), and between POCC and abundance of Urothoidae ($\rho = 0.832$, $p < 0.05$), recorded at the reference plot before/after the tuna penning activities; and (iii) between MSGS and abundance of Phoxocephalidae ($\rho = 0.766$, $p < 0.05$) recorded overall from the impacted/reference plots before the tuna penning activities (Table 4.4).

**Table 4.4** BEST results showing the sediment-physico chemical variable or combination thereof that best explains the observed variation in attributes of the polychaete and amphipod assemblages, and polychaete/amphipod index, recorded at impacted and reference plots at the three farms overall during the study period, and at the three farms before and after initiation of the tuna penning activities overall at impacted and reference plots. Level of significance set at 0.05. ρ-value = Spearman's rank correlation coefficient, Best Exp Var = Best Explanatory Variable, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', Im = impacted plot, Re = reference plot, Be = before, Af = after, NI = number of individuals; Polychaete indicator taxon 1 = Maldanidae, 2 = Paraonidae, 3 = Glyceridae; Amphipod indicator taxon 1 = Lysianassidae, 2 = Urothoidae, 3 = Phoxocephalidae; NFa = number of families, ShW = Shannon-Wiener diversity, AsC = assemblage composition, MSGS = mean sediment grain size, PONC = percent organic nitrogen content, POCC = percent organic carbon content, BOPA-FF = BOPA-Fish farming family index, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | | | NEF | | SEF 1 | | SEF 2 | |
|---|---|---|---|---|---|---|---|---|
| | | | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| Polychaetes | Im | NI 1 | -0.055, ns | PONC | 0.811, ns | MSGS | 0.516, ns | PONC |
| | | NI 2 | 0.159, ns | POCC | 0.370, ns | PONC | 0.671, * | PONC |
| | | NI 3 | 0.290, ns | PONC | 0.819, ns | POCC | 0.086, ns | POCC, PONC |
| | | NFa | 0.525, ns | PONC | 0.852, * | POCC | 0.215, ns | PONC |
| | | ShW | 0.607, * | MSGS, POCC | 0.921, *** | POCC, PONC | 0.047, ns | PONC |
| | | AsC | 0.661, * | POCC, PONC | 0.413, ns | PONC | 0.491, ns | PONC |
| | Re | NI 1 | 0.488, ns | MSGS, POCC | 0.379, ns | POCC, PONC | 0.865, * | MSGS, POCC |
| | | NI 2 | 0.592, ns | MSGS | 0.336, ns | POCC, PONC | 0.832, * | POCC |
| | | NI 3 | 0.722, ns | MSGS | 0.604, * | MSGS, POCC | -0.145, ns | PONC |
| | | NFa | -0.034, ns | PONC | 0.036, ns | MSGS, POCC | 0.313, ns | POCC, PONC |
| | | ShW | 0.014, ns | POCC | 0.157, ns | POCC, PONC | -0.107, ns | MSGS, PONC |
| | | AsC | 0.346, ns | MSGS, PONC | 0.207, ns | POCC | -0.304, ns | MSGS |
| Amphipods | Im | NI 1 | -0.027, ns | MSGS | 0.683, ns | MSGS, POCC | 0.013, ns | POCC |
| | | NI 2 | 0.300, ns | MSGS, POCC | -0.020, ns | PONC | 0.461, ns | POCC, PONC |
| | | NI 3 | -0.077, ns | POCC | -0.020, ns | PONC | 0.470, ns | POCC, PONC |
| | | NFa | 0.569, ns | MSGS, POCC | 0.400, ns | PONC | 0.451, ns | PONC |
| | | ShW | 0.530, ns | MSGS, POCC | 0.854, ns | POCC, PONC | 0.249, ns | PONC |

**Table 4.4** Continued

| | | | NEF | | SEF 1 | | SEF 2 | |
|---|---|---|---|---|---|---|---|---|
| | | | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| Amphipods | Im | AsC | 0.677, ns | POCC | 0.386, ns | PONC | 0.571, ns | PONC |
| | Re | NI 1 | 0.361, ns | MSGS | 0.286, ns | POCC | 0.013, ns | MSGS |
| | | NI 2 | 0.419, ns | PONC | 0.379, ns | POCC | -0.134, ns | MSGS |
| | | NI 3 | 0.283, ns | MSGS | 0.123, ns | POCC | 0.392, ns | MSGS, POCC |
| | | NFa | 0.073, ns | POCC | 0.095, ns | POCC | 0.068, ns | MSGS, POCC |
| | | ShW | 0.286, ns | PONC | 0.146, ns | POCC | 0.229, ns | MSGS, POCC |
| | | AsC | 0.557, ns | MSGS, PONC | 0.064, ns | POCC | 0.011, ns | MSGS |
| BOPA-FF | Im | | 0.479, ns | MSGS, POCC | 0.743, ns | POCC, PONC | 0.410, ns | PONC |
| | Re | | 0.125, ns | MSGS | 0.754, * | MSGS, POCC | 0.525, ns | MSGS, POCC |
| Polychaetes | Be | NI 1 | -0.013, ns | MSGS | 0.285, ns | MSGS, POCC | 0.669, ns | POCC |
| | | NI 2 | -0.120, ns | POCC | 0.332, ns | POCC | 0.680, ns | POCC, PONC |
| | | NI 3 | -0.209, ns | POCC | 0.931, ** | PONC | -0.083, ns | MSGS, POCC |
| | | NFa | 0.361, ns | PONC | 0.441, ns | PONC | 0.628, ns | MSGS |
| | | ShW | 0.136, ns | PONC | 0.214, ns | POCC | 0.189, ns | MSGS |
| | | AsC | 0.163, ns | PONC | 0.811, * | POCC | 0.632, ns | POCC, PONC |
| | Af | NI 1 | 0.022, ns | MSGS | 0.467, ns | PONC | 0.495, ns | MSGS, POCC |
| | | NI 2 | 0.343, ns | MSGS, POCC | 0.527, ns | POCC, PONC | 0.729, ns | PONC |
| | | NI 3 | 0.052, ns | MSGS, POCC | 0.622, ns | POCC | 0.090, ns | MSGS |
| | | NFa | 0.521, ns | MSGS, POCC | 0.515, ns | POCC, PONC | 0.703, ns | POCC, PONC |
| | | ShW | 0.668, * | MSGS, POCC | 0.780, ns | POCC, PONC | 0.206, ns | PONC |
| | | AsC | 0.718, * | POCC, PONC | 0.304, ns | POCC | 0.643, ns | POCC |
| Amphipods | Be | NI 1 | -0.182, ns | MSGS,PONC | 0.381, ns | POCC | 0.358, ns | POCC |
| | | NI 2 | 0.619, ns | MSGS,PONC | 0.185, ns | POCC | 0.088, ns | POCC |

**Table 4.4** Continued

| | | | NEF | | SEF 1 | | SEF 2 | |
|---|---|---|---|---|---|---|---|---|
| | | | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| Amphipods | Be | NI 3 | 0.683, ns | PONC | 0.386, ns | POCC | 0.766, * | MSGS |
| | | NFa | 0.586, ns | PONC | -0.185, ns | MSGS | 0.702, ns | PONC |
| | | ShW | 0.568, ns | MSGS | 0.143, ns | POCC | 0.693, ns | PONC |
| | | AsC | 0.349, ns | PONC | 0.379, ns | MSGS, POCC | 0.671, * | MSGS, POCC, PONC |
| | Af | NI 1 | 0.459, ns | MSGS, POCC | 0.815, * | POCC | 0.130, ns | MSGS |
| | | NI 2 | 0.030, ns | MSGS, POCC | 0.192, ns | MSGS, POCC | -0.056, ns | PONC |
| | | NI 3 | 0.170, ns | MSGS, POCC | 0.274, ns | MSGS | -0.139, ns | POCC |
| | | NFa | 0.613, * | MSGS, POCC | 0.522, ns | MSGS, POCC | -0.165, ns | MSGS |
| | | ShW | 0.810, ** | MSGS, POCC | 0.871, ** | POCC | -0.086, ns | POCC |
| | | AsC | 0.776, * | POCC | 0.652, ns | PONC | -0.068, ns | MSGS |
| BOPA-FF | Be | | 0.689, ns | MSGS, PONC | 0.579, ns | PONC | 0.607, ns | MSGS, POCC |
| | Af | | 0.404, ns | MSGS, POCC | 0.700, ns | MSGS, POCC, PONC | 0.114, ns | POCC |

### 4.3.2 Multivariate data analyses

#### 4.3.2(i) Macroinvertebrate assemblages

The PCO ordination accounted for 63.2 % and 50.9 % of the total variation recorded respectively in the abundance of polychaete and amphipod families, and showed clear grouping of samples (Figure 4.5). Samples collected from the SEF 1 impacted and reference plots following the tuna penning activities were distinctly separated from all other samples by the first PC axis (Figure 4.5). The second PC axis separated the remaining samples into two distinct groups: a group of samples collected from the NEF impacted plots after the tuna penning activities, and a group of samples collected from the NEF, the SEF 1 and the SEF 2 impacted and reference plots before tuna penning commenced, and from the NEF reference plot and SEF 2 impacted and reference plots following initiation of the activities (Figure 4.5).

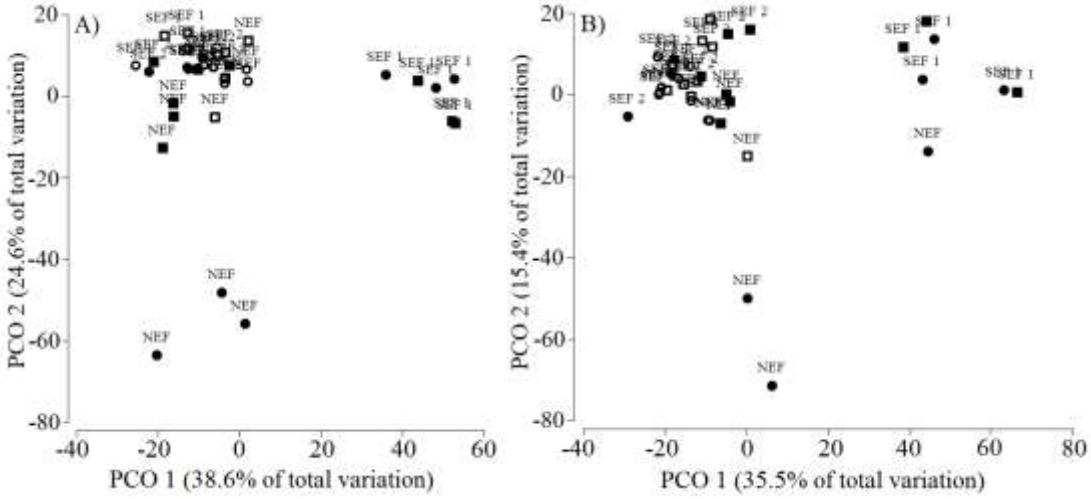


**Figure 4.5** PCO plots calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data: (a) polychaetes and (b) ampihipods for the impacted (circle) and reference (square) plots before initiation of tuna penning activities (unshaded), and afterwards (shaded), at the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1), and southeastern 'Farm 2' (SEF 2).

Multivariate PERMANOVA showed that the square root estimate of 'BA x Lo x Pl' as a component of variation in abundance of polychaetes and amphipods (respectively 24.16 & 24.46), was small compared with that of 'BA x Lo' (respectively 37.15 &

25.84) and the residual (respectively 30.73 & 43.05) (Table 4.4). Both PERMANOVA and PERMDISP tests indicated significant differences ($p < 0.0001$) in the polychaete and amphipod assemblage composition for 'BA x Lo x Pl' (Table 4.5).

**Table 4.5** Results of the four-factor, multivariate PERMANOVA and PERMDISP tests calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data for polychaetes and amphipods, with *a posteriori* comparisons for the significant third-order interaction term. Variables included in the analyses are fourth-root transformed family abundance values. Level of significance set at 0.05. Df = Degrees of freedom, Sq Rt Est = Square Root Estimate of Variation, p(PERM) = Permutational p-value, Im = impacted plot, Re = reference plot, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', Be = before, Af = after, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

| | | | Polychaetes | | | Amphipods | |
|---|---|---|---|---|---|---|---|
| | | | PERMANOVA | PERMDISP | PERMANOVA | | PERMDISP |
| Source of Variation | | df | Sq Rt Est | p(PERM) | p(PERM) | Sq Rt Est | p(PERM) | p(PERM) |
| Before/After = BA | | 1 | 14.92 | **** | ** | 23.83 | **** | *** |
| Location = Lo | | 1 | 8.89 | *** | ns | 5.48 | ns | ns |
| Plot = Pl | | 2 | 20.45 | **** | ** | 19.41 | **** | ns |
| BA x Lo | | 2 | 37.15 | **** | ** | 25.84 | **** | *** |
| BA x Pl | | 1 | 11.16 | ** | ** | 11.91 | ** | *** |
| Pl x Lo | | 2 | 15.34 | *** | **** | 18.14 | **** | **** |
| BA x Lo x Pl | | 2 | 24.16 | **** | **** | 24.46 | **** | **** |
| Site = Si (BA x Lo x Pl) | | 24 | 11.56 | ** | ns | 10.08 | ns | ns |
| Residual | | 72 | 30.73 | | | 43.05 | | |
| Total | | 107 | | | | | | |
| *Pair-wise tests -: 'BA x Lo x Pl'* | | | | | | | | |
| Im | NEF | Be, Af | | *** | *** | | * | *** |
| | SEF 1 | | | *** | ns | | *** | * |
| | SEF 2 | | | * | ns | | * | ** |
| Re | NEF | | | ** | **** | | ns | ns |
| | SEF 1 | | | *** | ** | | *** | ns |
| | SEF 2 | | | ns | * | | * | **** |
| Be | NEF | Im, Re | | ns | ns | | ns | ns |
| | SEF 1 | | | * | **** | | ** | ns |
| | SEF 2 | | | ns | ns | | * | ** |
| Af | NEF | | | *** | ns | | ** | **** |
| | SEF 1 | | | ns | ns | | ns | ns |
| | SEF 2 | | | ns | ns | | ns | ns |
| Be | Im | NEF, SEF 1 | | * | * | | ns | ns |
| | | NEF, SEF 2 | | * | ** | | * | * |
| | | SEF 1, SEF 2 | | ns | * | | * | ** |
| | Re | NEF, SEF 1 | | ** | **** | | * | ns |
| | | NEF, SEF 2 | | * | * | | * | ns |
| | | SEF 1, SEF 2 | | * | * | | ns | ns |

**Table 4.5** Continued

| Source of Variation | | df | Polychaetes PERMANOVA Sq Rt Est | Polychaetes PERMANOVA p(PERM) | Polychaetes PERMDISP p(PERM) | Amphipods PERMANOVA Sq Rt Est | Amphipods PERMANOVA p(PERM) | Amphipods PERMDISP p(PERM) |
|---|---|---|---|---|---|---|---|---|
| *Pair-wise tests -:* | | *'BA x Lo x Pl'* | | | | | | |
| Af | Im | NEF, SEF 1 | | *** | ** | | * | * |
| | | NEF, SEF 2 | | *** | ns | | ** | ** |
| | | SEF 1, SEF 2 | | *** | ns | | *** | ns |
| | Re | NEF, SEF 1 | | *** | ns | | *** | ns |
| | | NEF, SEF 2 | | ** | * | | ** | ** |
| | | SEF 1, SEF 2 | | ** | ** | | ** | **** |

Pair-wise tests indicated significant differences (see Table 4.5) in the assemblage composition of polychaetes and amphipods at the NEF, the SEF 1, and the SEF 2 impacted and reference plots following initiation of tuna penning activities (Table 4.5). The polychaete and amphipod assemblage composition differed significantly ($p_{\text{Polychaetes PERMANOVA}} < 0.001$, $p_{\text{Amphipods PERMANOVA}} < 0.01$, $_{\text{PERMDISP}} < 0.0001$) between the NEF impacted and reference plots in the same period. Pair-wise tests also showed significant difference in the amphipod assemblage composition between impacted plots of the NEF and the SEF 1 ($p < 0.05$), and between reference plots of the SEF 1 and the SEF 2 ($p < 0.001$) (Table 4.4). PERMANOVA also indicated significant difference ($p < 0.01$) in the polychaete assemblage composition for 'Si(BA x Lo x Pl)' (Table 4.5).

SIMPER analysis indicated a high dissimilarity in the abundance of polychaetes and amphipods at the NEF impacted plot from before to after tuna penning activities (respectively 81.52 % and 90.54 %), and between the NEF impacted and reference plots following tuna penning activities (respectively 71.19% and 90.82%) (Table 4.6). The top polychaete taxa that contributed most to this dissimilarity (in terms of number of individuals) were Capitellidae; which increased in abundance at the impacted plot after the tuna penning activities; and Paraonidae, Terebellidae, and Glyceridae; which decreased in abundance at the impacted plot after the tuna penning activities (Table 4.6). The top amphipod taxa that contributed most to this dissimilarity (i.e.

Phoxocephalidae, Maeridae, Urothoidae, Lysianassidae, and Photidae) decreased in abundance at the impacted plot after the tuna penning activities (Table 4.6).

At the SEF 1, SIMPER analysis indicated high dissimilarity in the abundance of polychaetes and amphipods at both the impacted (respectively 82.48 % and 91.60 %) and reference (respectively 79.01% and 90.37 %) plots from before to after the tuna penning activities (Table 4.6). The polychaete taxa that contributed most to this dissimilarity were: (i) Flabelligeridae and Phyllodocidae, which increased in abundance, and Glyceridae; which decreased in abundance, at the impacted plot, and (ii) Maldanidae, Glyceridae, and Sabellidae, which decreased in abundance at the reference plot; following initiation of the tuna penning activities (Table 4.6). The amphipod taxa that contributed most to this dissimilarity; i.e. Urothoidae, Phoxocephalidae, and Lysianassidae, at the impacted plot, and Lysianassidae, Philantidae, and Photidae at the reference plot; decreased in abundance in the same period (with the exception of Philantidae, which increased in abundance at the reference plot) (Table 4.6). The dissimilarity in the abundance of polychaetes and amphipods between the SEF 1 impacted and reference plots was low both before (respectively 44.71 % and 55.69 %) and after (respectively 48.59 % and 69.61 %) the tuna penning activities (Table 4.6).

At the SEF 2, SIMPER analysis indicated low dissimilarity in the abundance of polychaetes at the impacted (47.01 %) and reference (53.15 %) plots from before to after the tuna penning activities, and between the impacted and reference plots both before (45.09 %) and after (54.70 %) tuna penning activities (Table 4.6). SIMPER also indicated a high dissimilarity in the abundance of amphipods at the SEF 2 reference plot following initiation of the tuna penning activities (79.81 %), and between impacted and reference plots in the same period (76.61 %). The amphipod taxa that contributed most to this dissimilarity (i.e. Cheirocratidae, Maeridae, Lysianassidae, and Urothoidae), decreased in abundance at the SEF 1 reference plot following initiation of tuna penning activities (Table 4.6).

**Table 4.6** Results of SIMPER analysis calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data for polychaetes and amphipods, showing the top three families (in terms of abundance) contributing to the dissimilarity of benthic assemblages recorded from before to after the tuna penning activities at the impacted and reference plots, and between the impacted and reference plots before and after the tuna penning activities. Avg Diss (%) = Average Dissimilarity (%); Avg Abun = Average Abundance, Contrib % = Contribution (%), NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', Im = impacted plot, Re = reference plot, Be = before farming, Af = after farming

| | | | Polychaetes | | | | | Amphipods | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Avg Abun | | | | | Avg Abun | | |
| Levels | | Pairs | Avg Diss % | Family | x | y | Contrb % | Avg Diss % | Family | x | y | Contrb % |
| NEF | Im | | 81.52 | Capitellidae | 0.77 | **3.53** | 26.23 | 90.54 | Phoxocephalidae | **1.32** | 0.22 | 23.84 |
| | | | | Paraonidae | **1.21** | 0.49 | 8.73 | | Maeridae | **0.68** | 0.00 | 18.50 |
| | | | | Terebellidae | **0.96** | 0.00 | 8.28 | | Urothoidae | **0.87** | 0.00 | 16.65 |
| | Re | Be (x), Af (y) | 63.37 | Glyceridae | 0.11 | **1.52** | 10.38 | 53.06 | Photidae | 0.00 | **1.21** | 17.52 |
| | | | | Hesionidae | 0.00 | **1.14** | 8.31 | | Urothoidae | 0.79 | **1.27** | 12.82 |
| | | | | Sabellidae | 0.00 | **1.10** | 7.77 | | Ampeliscidae | **0.87** | 0.79 | 10.57 |
| | Be | | 55.86 | Lumbrineridae | **0.91** | 0.40 | 8.55 | 53.96 | Maeridae | **0.68** | 0.38 | 15.17 |
| | | | | Terebellidae | **0.96** | 0.54 | 8.43 | | Urothoidae | **0.87** | 0.79 | 14.68 |
| | | | | Capitellidae | **0.77** | 0.61 | 8.14 | | Phoxocephalidae | 1.32 | **1.51** | 14.43 |
| | Af | Im (x), Re (y) | 71.19 | Capitellidae | **3.53** | 1.09 | 18.46 | 90.82 | Lysianassidae | 0.11 | **1.41** | 17.39 |
| | | | | Paraonidae | 0.49 | **1.96** | 11.19 | | Urothoidae | 0.00 | **1.27** | 16.62 |
| | | | | Glyceridae | 0.28 | **1.52** | 9.30 | | Photidae | 0.00 | **1.21** | 16.04 |
| SEF 1 | Im | | 82.48 | Flabelligeridae | 0.00 | **2.44** | 14.87 | 91.60 | Urothoidae | **1.61** | 0.00 | 16.33 |
| | | | | Phyllodocidae | 0.28 | **1.57** | 8.77 | | Phoxocephalidae | **1.17** | 0.00 | 12.07 |
| | | | | Glyceridae | **1.38** | 0.00 | 8.66 | | Lysianassidae | **0.96** | 0.00 | 9.93 |
| | Re | Be (x), Af (y) | 79.01 | Maldanidae | **2.45** | 0.00 | 13.55 | 90.37 | Lysianassidae | **2.09** | 0.11 | 16.19 |
| | | | | Glyceridae | **1.64** | 0.00 | 9.09 | | Philantidae | 0.11 | **1.29** | 10.02 |
| | | | | Sabellidae | **1.55** | 0.38 | 6.72 | | Photidae | **1.11** | 0.00 | 8.87 |

**Table 4.6** Continued

| | | | | Polychaetes | | | | Amphipods | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Avg Abun | | | | | Avg Abun | | |
| Levels | | Pairs | Avg Diss % | Family | x | y | % Contrb | Avg Diss % | Family | x | y | Avg Diss % |
| SEF 1 | Be | Im (x), Re (y) | 44.71 | Maldanidae | 1.33 | **2.45** | 8.91 | 55.69 | Lysianassidae | 0.96 | **2.09** | 12.04 |
| | | | | Sabellidae | 0.49 | **1.55** | 8.79 | | Photidae | 0.15 | **1.11** | 10.85 |
| | | | | Syllidae | 0.32 | **0.87** | 6.49 | | Urothoidae | **1.61** | 0.93 | 9.48 |
| | | | 48.59 | Flabelligeridae | 2.44 | **0.75** | 24.85 | 69.61 | Philantidae | **0.62** | 1.29 | 18.80 |
| | | | | Terebellidae | 0.60 | **1.01** | 10.84 | | Calappidae | 0.35 | **1.05** | 17.87 |
| | | | | Paraonidae | **0.80** | 0.72 | 8.82 | | Aoridae | **0.83** | 0.60 | 15.27 |
| SEF 2 | Im | Be (x), Af (y) | 47.01 | Nereididae | **1.29** | 0.28 | 8.63 | 58.21 | Photidae | **1.65** | 0.22 | 15.25 |
| | | | | Sabellidae | **0.92** | 0.13 | 7.07 | | Ampeliscidae | **1.49** | 0.33 | 12.52 |
| | | | | Maldanidae | 0.58 | **0.95** | 6.26 | | Phoxocephalidae | **1.24** | 0.43 | 10.38 |
| | Re | | 53.15 | Sabellidae | **1.55** | 0.45 | 7.45 | 79.81 | Cheirocratidae | **1.24** | 0.11 | 13.27 |
| | | | | Eunicidae | **1.67** | 0.88 | 6.24 | | Maeridae | **1.31** | 0.29 | 12.38 |
| | | | | Syllidaae | **1.28** | 0.77 | 5.95 | | Lysianassidae | **1.11** | 0.24 | 10.41 |
| | Be | Im (x), Re (y) | 45.09 | Eunicidae | 0.51 | **1.67** | 7.92 | 51.76 | Photidae | **1.65** | 0.11 | 14.44 |
| | | | | Syllidaae | 0.26 | **1.28** | 7.13 | | Urothoidae | **1.87** | 0.72 | 10.75 |
| | | | | Scalibregmatidae | 0.00 | **0.87** | 6.03 | | Maeridae | 0.39 | **1.31** | 9.37 |
| | Af | | 54.70 | Paraonidae | **1.14** | 0.80 | 6.40 | 76.61 | Urothoidae | **1.31** | 0.70 | 19.25 |
| | | | | Cirratulidae | **1.00** | 0.49 | 5.81 | | Lysianassidae | **0.75** | 0.24 | 12.91 |
| | | | | Maldanidae | 0.95 | **1.12** | 5.80 | | Maeridae | **0.42** | 0.29 | 7.98 |

*A posteriori* PERMANOVA indicated a significant difference for the interaction term 'BA x Pl' in the abundance of: (i) Capitellidae and Photidae at the NEF ($p < 0.01$); (ii) Phyllodocidae, Sabellidae, Philantidae and Photidae ($p < 0.05$ except $p_{Phyllodocidae} < 0.01$) at the SEF 1; and (iii) Maeirdae at the SEF 2 ($p < 0.05$) (Table 4.7). Pair-wise tests showed that the abundance of Capitellidae increased significantly ($p < 0.05$) at the NEF impacted plot following the tuna penning activities, while the abundance of Photidae increased significantly ($p < 0.05$) at the NEF reference plot in the same period (Table 4.7).

*A posteriori* PERMANOVA also showed a significant difference ($p < 0.05$) in 'BA' for the abundance of Terebellidae and Maeridae at the NEF (Table 4.6). Pair-wise tests showed that the abundance of Terebellidae and Maeridae at the NEF impacted/reference plots decreased significantly ($p < 0.01$) following the tuna penning activities (Table 4.7).

**4.3.2(ii) Sediment physico-chemical attributes**

PCO ordination explained 73.6% of the total variation in sediment physico-chemical data, and indicated clear separation between sediment samples (Figure 4.6). Sediment samples collected from the NEF impacted plot following initiation of tuna penning activities were distinctly separated from all other samples by the first PC axis. Other distinct groups of sediment samples compromised: (i) samples collected from the SEF 1 impacted plot and the SEF 2 reference plot after tuna penning; (ii) samples collected from the SEF 2 reference plot before tuna penning commenced; and (iii) samples collected before tuna penning commenced from the NEF, the SEF 1 and the SEF 2 impacted and reference plots, and from the NEF and SEF 1 reference plots after initiation of tuna penning activities (Figure 4.6).

Multivariate PERMANOVA showed that the square root estimates of the factors, interaction terms, and residual variation, as components of variation in sediment physico-chemical data, were small or negative ($0.65196_{BA}$ to $-0.40249_{Pl}$) (Table 4.8). PERMDISP indicated a significant difference ($p < 0.01$) in sediment physico-chemical

**Table 4.7** Results of the three-factor *a posteriori* univariate PERMANOVA for number of individuals of taxa that contributed most to the dissimilarity in polychaete and amphipod assemblages at the impacted and reference plots from before to after tuna penning activities, and between the impacted and reference plots before and after initiation of the tuna penning activities, for each farm; with *a posteriori* pair-wise comparisons for the significant factors and second-order interaction term. Level of significance set at 0.05. Df = Degrees of freedom, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', Cap = Capitellidae, Ter = Terebellidae, Mae = Maeridae, Pho = Photidae, Fla = Flabelligeridae, Phy = Phyllodocidae, Sab = Sabellidae, Phi = Philantidae, Im = impacted plot, Re = reference plot, Be = before, Af = after, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | | | NEF | | | | | SEF1 | | | | SEF2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | | df | Cap | Ter | Mae | Pho | Fla | Phy | Sab | Phi | Pho | Mae |
| Before/After = BA | | 1 | ** | * | * | * | ns | ** | * | ** | * | ns |
| Plot = Pl | | 1 | ** | ns | ns | * | ns | ** | * | * | * | ns |
| BA x Pl | | 1 | ** | ns | ns | ** | ns | ** | * | * | * | * |
| Site = Si (BA x Pl) | | 8 | ** | * | ns | ns | ns | ns | *** | * | ns | ns |
| Residual | | 24 | | | | | | | | | | |
| Total | | 35 | | | | | | | | | | |
| *Pair-wise tests -: 'BA x Pl'* | | | | | | | | | | | | |
| Im | Be, Af | | < * | - | - | ns | - | < ** | ns | ns | ns | ns |
| Re | | | ns | - | - | < * | - | < *** | ns | < * | > * | ns |
| Be | Im, Re | | ns | - | - | ns | - | ns | ns | ns | < * | ns |
| Af | | | > * | - | - | < * | - | < ** | ns | < * | ns | ns |
| *Pair-wise tests -: 'BA' x 'Pl'* | | | | | | | | | | | | |
| Be, Af | | | - | * > | * > | - | - | - | - | - | - | - |
| Im, Re | | | - | - | - | - | - | - | - | - | - | - |

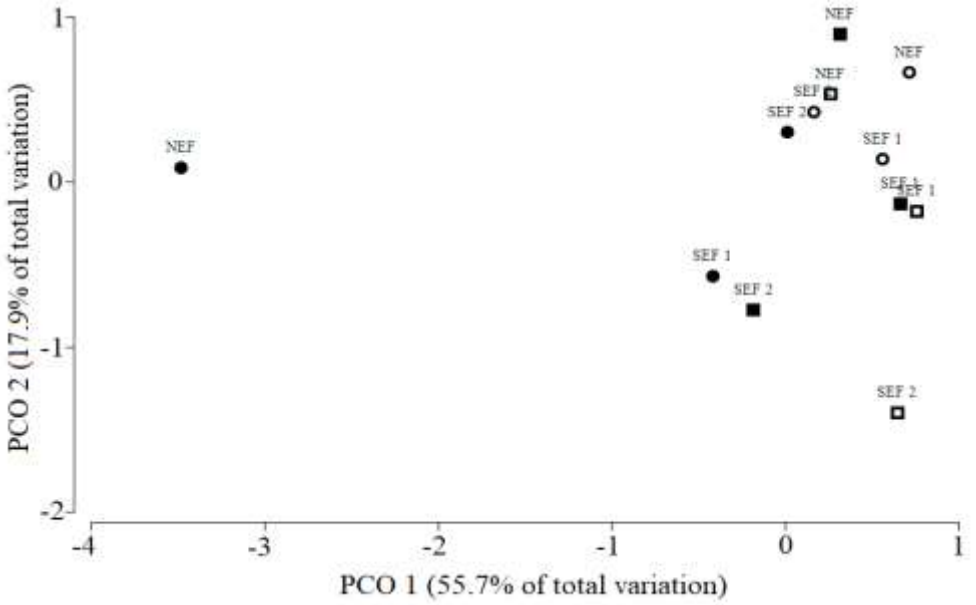


**Figure 4.6** PCO plot calculated from a Euclidean similarity matrix of normalised sediment physico-chemical data recorded from the impacted (circle) and reference (square) plots before initiation of tuna penning activities (unshaded), and afterwards (shaded), at the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1), and southeastern 'Farm 2' (SEF 2).

data for 'BA x Pl x Lo', while PERMANOVA did not indicate any significant difference (Table 4.8). Pair-wise tests showed that sediment physico-chemical data differed significantly ($p < 0.01$) at the NEF impacted plot following initiation of the tuna penning activities, and between the impacted and reference plots of the NEF ($p < 0.01$) and the SEF 1 ($p < 0.05$), and between the NEF and the SEF 1, and the SEF 2, at the impacted plot ($p < 0.05$), and the NEF and the SEF 2 at the reference plot ($p < 0.05$), in the same period (Table 4.8).

### 4.3.2(iii) Relationship between sediment attributes and macroinvertebrates

BEST analysis showed a significant correlation between: (i) a combination of POCC and PONC, and polychaete abundance recorded overall from the NEF impacted/reference plots following initiation of tuna penning activities ($\rho = 0.718$, $p < 0.05$), and overall at the NEF impacted plot during the study period ($\rho = 0.661$, $p < 0.05$); and (ii) between POCC and amphipod abundance recorded overall at the NEF impacted and reference plots following initiation of tuna penning activities ($\rho = 0.776$, $p < 0.05$) (Table 4.4).

**Table 4.8** Results of the three-factor PERMANOVA and PERMDISP tests calculated from a Euclidean similarity matrix of normalised sediment physico-chemical data, with *a posteriori* pair-wise comparisons for the significant third order interaction term. Variables included in the analyses are normalised mean sediment grain size, percent organic carbon content, and percent organic nitrogen content of the sediment. Level of significance set at 0.05. Df = Degrees of freedom, Sq Rt Est = Square Root Estimate of Variation, p(PERM) = Permutationl p-value, Be = before, Af = after, Im = impacted plot, Re = reference plot, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', ns = not significant, * = $p < 0.05$, ** = $p < 0.01$

| | | PERMANOVA | | PERMDISP |
|---|---|---|---|---|
| Source of Variation | df | Sq Rt Est | p(PERM) | p(PERM) |
| Before/After = BA | 1 | 0.65196 | ** | * |
| Location = Lo | 2 | 0.26507 | ns | ns |
| Plot = Pl | 1 | 0.40259 | ns | ns |
| BA x Lo | 2 | -0.05884 | ns | ns |
| BA x Pl | 1 | 0.63647 | ns | * |
| Pl x Lo | 2 | 0.60018 | ns | * |
| BA x Lo x Pl | 2 | 0.90276 | ns | ** |
| Residual | 24 | 1.5112 | | |
| Total | 35 | | | |
| *Pair-wise PERMDISP tests -: 'BA x Lo x Pl'* | | | | |
| | | | Im | Re |
| NEF | Be, Af | | ** | ns |
| SEF 1 | | | ns | ns |
| SEF 2 | | | ns | ns |
| | | | Be | Af |
| NEF | Im, Re | | ns | ** |
| SEF 1 | | | ns | * |
| SEF 2 | | | ns | ns |
| Im | NEF, SEF 1 | | * | ns |
| | NEF, SEF 2 | | ns | * |
| | SEF 1, SEF 2 | | ns | * |
| Re | NEF, SEF 1 | | ns | ns |
| | NEF, SEF 2 | | ns | * |
| | SEF 1, SEF 2 | | ns | ns |

BEST analysis also showed a significant correlation: (i) between POCC and polychaete abundance ($\rho = 0.811$, $p < 0.05$) recorded overall from the SEF 1 impacted/reference plots before tuna penning commenced; and (ii) between a combination of MSGS, POCC and PONC, and amphipod abundance ($\rho = 0.671$, $p < 0.05$), recorded overall from the SEF 2 impacted/reference plots following initiation of tuna penning activities (Table 4.4).

## 4.4 Discussion

The present results show that tuna penning activities resulted in alterations to the benthic invertebrate assemblages, with the observed changes probably resulting from the accumulation of uneaten feed-fish on the seabed below the tuna cages (Mangion *et al.*, 2014). Values of the biological parameters varied spatially, particularly at the scale of location (km). Previous studies at Mediterranean fish farms recorded high spatial variation in attributes of peracarid crustacean assemblages (Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011) in the vicinity of fish cages. Consideration of spatial variation in ecological studies that utilise a hierarchical nested design is important since the power of statistical tests is reduced (see Morrisey, 1992) when small scale variation is larger than the variation at higher spatial scales (e.g. Anderson *et al*., 2005; Chapman, Tolhurst, Murphy, & Underwood, 2010; Fernandez-Gonzalez *et al*., 2013; Fraschetti, Terlizzi, & Benedetti-Cecchi, 2005). In the present hierarchical study design, the power of statistical tests to detect observed differences in attributes of the benthic assemblage was increased (see Morrisey, 1992) by setting location as a fixed factor, rather than as a random factor nested within the higher scale of impacted/reference plot.

Studies at other Mediterranean tuna farms reported a low diversity of benthic assemblages below fish cages during the farming season (Jahani *et al*., 2012; Mangion *et al*., 2014; Marin *et al*., 2007; Vita & Marin, 2007), and elevated values of the ratio: polychaete/amphipod abundance (BOPA) (Jahani *et al*., 2012). However, other workers found no significant influence of tuna penning on phyisco-chemical parameters in the water column and sediment (Aksu *et al*., 2016), and benthic assemblages (Moraitis *et al*., 2013), which was attributed to high exposure; hence to a high energy environment that helped dispersal of organic matter generated at the farm; and to controlled feeding (Aksu *et al*., 2016; Moraitis *et al*., 2013). The effects of fish farm wastes on seabed habitats are determined by local environmental characteristics, such as bottom type, water depth, exposure and bottom currents, as well as the farms' feed management regime (Borja *et al*., 2009c; Tomassetti *et al*., 2009). Therefore, differences in the level and spatial extent of potential adverse environmental impacts

of tuna penning are expected between sites having different environmental characteristics. The three tuna farms investigated in the present study differed in size, stocking density and feed management, as well as their location; hence one would expect differences in the magnitude and spatial extent of potential adverse environmental impact among them.

Spatial variation in the influence of tuna penning on the polychaete and amphipod assemblages was significant at the scale of location. Furthermore, the number of polychaete families was significantly lower, and values of the polychaete/amphipod ratio were significantly higher, at the impacted plot of the northeastern farm; the macroinvertebrate assemblage composition was characterised by significantly elevated abundance values of opportunistic capitellid polychaetes (e.g. Borja *et al*., 2000), decreased abundance of sensitive Paraonidae polychaetes (e.g. Martinez-Garcia *et al*., 2013) and amphipod taxa (e.g. Fernandez-Gonzalez & Sanchez-Jerez, 2011), and by 'Bad' EQS. Overall, the results for the NEF were distinct from those for the southeastern farms. Concomitantly, sediment physico-chemical attributes changed significantly at the impacted plot; namely a significant increase in POCC; while levels of sediment feed-fish bone content below fish cages at the northeastern farm were elevated compared with the southeastern farms. In the present study, the northeastern farm had the largest annual fish holding capacity compared with the other two farms. Borja *et al*. (2009c) previously reported that benthic ecological quality was better at fish farm sites that had a lower total annual production, which is in agreement with the present results. The sediment MSGS, POCC, and PONC or combinations thereof, were significantly correlated with the diversity and assemblage composition of polychaete taxa, and with the number, diversity, and assemblage composition of amphipod taxa recorded overall at the impacted plot of the northeastern farm and at the northeastern farm after the tuna penning activities.

The influence of tuna penning on benthic habitat at the impacted plots of the southeastern farms was indicated by a significant decrease in the number of amphipod families, and the significant influence of sediment POCC and PONC on the abundance and diversity of polychaete and amphipod families. The elevated levels of sediment

FFBC below tuna cages of southeastern ‘Farm 1’ compared with southeastern ‘Farm 2’, and the ‘Moderate’ EQS recorded from the impacted plot of southeastern ‘Farm 1’, indicate that the influence of tuna penning on benthic habitat present in the immediate vicinity of southeastern ‘Farm 2’, which retained ‘Good’ EQS, was not as large. Sediment samples collected from below the cages of southeastern ‘Farm 2’ after the tuna penning activities appeared similar to those collected before initiation of the activities, and differed significantly from the other ‘impacted’ samples in the same period.

The level of tuna penning activities and feed management regime adopted at different tuna farms resulted in different levels of influence on sediment quality between cages within the same farm (Mangion *et al*., 2014), over and above the expected variation between different tuna farms. Given the potential high variation in biological attributes at small spatial scales, the pattern of influence of a fish farm on benthic biota at one site cannot be extrapolated to other farms at different sites (e.g. Fernandez-Gonzalez *et al*., 2013). Fernandez-Gonzalez *et al*. (2013) noted that spatial variation in attributes of benthic assemblages between different sites may be higher at fish farms compared with reference areas; this is characteristic of stressed assemblages (e.g. Stark, Riddle, & Simpson, 2003; Warwick & Clarke, 1993). For instance, for the same farm considered in the present study, i.e. the northeastern farm, Mangion *et al*. (2014) reported a significantly higher abundance of Capitellid polychaetes below cages, which varied at the scale of site. The present results showed that, when considering the three tuna farms, significant variation in the abundance of polychaetes (Maldanidae, Paraonidae) and amphipods (Lysianassidae, Urothoidae), and number of families, Shannon-Wiener diversity, and assemblage composition of polychaetes, was recorded at the scale of ‘site’, i.e. 100’s of meters.

The spatial extent of influence of fish farm waste on the marine environment will vary (Karakassis, Pitta, & Krom, 2005) from a localised level to a regional one that may extend several kilometres (Silvert, 1992). The influence of tuna penning on benthic habitat detected in the present study appears to exceed the largest spatial scale incorporated in the survey design, since some influence of the activity on

macroinvertebrate assemblages was detected c. 1 km away from the cages.

The macroinvertebrate assemblage composition at the reference plot located some 1 km away from southeastern 'Farm 1' appeared similar to that recorded in the immediate vicinity of the same farm following the tuna penning activities. Fernandez-Gonzalez *et al*. (2013) reported an influence of fish farming on spatial patterns of attributes of amphipod assemblages at spatial scales that varied from several meters to hundreds of kilometers. While a distance of c. 1 km would appear to be sufficient to minimize the influence of fish farm wastes on a reference area (Porello *et al*., 2005), the oligotrophic nature of the Mediterranean may render the benthic ecosystem more sensitive to organic input. Present results indicate that tuna penning at southeastern 'Farm 2' resulted in a significant decrease in the number of families and diversity of amphipods, high dissimilarity in amphipod assemblage composition charaterised by decreased amphipod abundances, and 'Moderate' EQS, at the reference plot located c. 1 km away from the tuna cages. Sediment physico-chemical data at this plot appeared similar to samples collected from the impacted plot of southeastern 'Farm 1' in the same period. The down-current orientation of the reference plot of southeastern 'Farm 2' with respect to both impacted plots of the two southeastern farms, may account for the influence of tuna penning observed there, since organic waste may have been transported to the reference plot via sea currents; the acquired sea current data indicated a predominantly southern current (189 º) having a mean velocity of 0.185 ms $^{-1}$ in the vicinity of the two southeastern farms. It is possible that other unidentified factors apart from ones related to the tuna penning activities, may have influenced the soft bottom habitat at the reference plot of southeastern 'Farm 2', although the changes recorded there are in all probability due to the tuna penning activities, since they coincide with the onset of tuna penning in the general area. Apart from the location and size of the farm, the magnitude and spatial extent of tuna penning impacts are also determined by a farm's specific feed management regime (Mangion *et al*., 2014).

The present results show that the magnitude of influence of tuna penning activities on benthic invertebrate assemblages varies significantly among different tuna penning locations having a different size of operations and local environmental and

oceanographic factors. The influence of tuna penning activities on benthic invertebrate assemblages was larger at the impacted plot of the largest tuna farm in terms of ABT holding capacity and production – compared with the other two smaller farms. On the other hand, the spatial extent of influence appeared to be largest at one of the southeastern farms ('Farm 2'), where the influence of tuna penning activities extended down-current in a southern direction, up to some 1 km away from fish cages; this may possibly reflect an 'additive effect' of the two southeastern farms, given that they were relatively close to each other (1 km apart). Taken together, these observations corroborate the expectation that the level and extent of influence of tuna penning activities on benthic habitat in the vicinity will be larger for farms having higher fish stocking density, in areas where sea currents are not strong. Furthermore, farms located relatively close to one another may result in added loading on the environment, leading to a larger spatial extent of environmental impact - this latter observation has implications for spatial planning of tuna penning activities, particularly given that many countries are moving toward establishing "Allocated Zones for Aquaculture" (AZA); see Sanchez-Jerez *et al*. (2016). Finally, the present findings also show that inclusion of multiple reference areas in monitoring programmes is important for assessing potential environmental impacts of tuna farms.

# CHAPTER 5

# SPATIAL PATTERNS IN BENTHIC MACROINVERTEBRATE ASSEMBLAGE STRUCTURE UNDER THE INFLUENCE OF TUNA PENNING

## Chapter 5.1 Introduction

Organic enrichment is a common cause of disturbance of macroinvertebrate assemblages associated with soft bottom habitat. One source of marine pollution is the coastal aquaculture industry, which introduces high levels of organic matter to the benthic ecosystem, leading to potentially adverse effects on macroinvertebrate assemblages associated with the seabed in the vicinity of fish farms (e.g. Claudet & Fraschetti, 2010; Fernandez-Gonzalez *et al*., 2013; Hall, Anderson, Holby, Kollberg, & Samuelsson, 1990; Hargrave *et al*., 1997; GESAMP, 1990; Wu, 1995).

The influence of fish farm wastes on the seabed varies with distance from the farm, as well as with water depth and the hydrodynamic regime of the area (Kalantzi & Karakassis, 2006). The horizontal distance from a sea-based fish farm is more important in influencing the structure of benthic macroinvertebrate assemblages present in the vicinity of the fish cages than depth and sediment type (Salvo, Mersereau, Hamoutene, Belley, & Dufour, 2017). Studies on the influence of fish farm wastes on soft bottom habitats report that benthic macroinvertebrate assemblages show a spatial pattern in species diversity that has been described by the Pearson-Rosenberg (P-R) 1978 model, as modified in later published works (Sanz-Lázaro & Marin, 2011). However, several studies aimed at assessing the influence of fish farming on benthic habitats and biotic assemblages do not report the peak in species diversity at intermediate levels of organic enrichment as described by the P-R model (Sanz-Lázaro & Marin, 2011). This is because of differences in the effects of fish farming on benthic habitat resulting from differences in the farmed species, total organic input to the marine environment, years of operation and feed management regime adopted at different fish farms. Furthermore, different fish farms are located at sites that have different exposure, sea current regime, water depth and sediment type (e.g. Borja *et al*., 2009c; Tomassetti *et al*., 2009). Additionally, in the case of the Mediterranean, benthic macroinvertebrate assemblages in this oligotrophic sea may respond differently to the introduction of organic wastes compared to places that are characterised by more mesotrophic ecosystems such as in the Atlantic (Karakassis *et al*., 2000).

Most studies on spatial patterns in benthic macroinvertebrate assemblages under the influence of fish farming have been carried out for salmon farms in northern Europe and northern America (e.g. Brown, Gowen, & Mclusky, 1987; Kupka-Hansen, Pittman, & Ervik, 1991; Kutti *et al*., 2007a; Nickell *et al*., 2003; Salvo *et al*., 2017; Weston, 1990) and for sea bass and sea bream farms in the Mediterranean (e.g. Di Marco *et al*., 2017; Fernandez-Gonzalez *et al*., 2013; Karakassis *et al*., 2000; Tomassetti *et al*., 2016). The environmental impacts of tuna penning differ from those of other intensive aquaculture activities in the Mediterranean due to the large size of the farmed fish, the use of feed-fish instead of processed feed, and the poor food conversion ratio, all of which result in higher organic loading of the benthic ecosystem below fish cages (Aguado-Giménez *et al*., 2006). Furthermore, the spatial extent of influence of the tuna penning activities is expected to be larger compared to that reported for other fish farms such as sea bass and sea bream aquaculture installations (e.g. Karakassis *et al*., 2000, 2002). While several studies have been carried out in the Mediterranean to assess the potential adverse effects of tuna farms on the species composition and structure of benthic macroinvertebrate assemblages present in the vicinity (Borg & Schembri, 2005; Holmer *et al*., 2008; Jahani *et al*., 2012; Mangion *et al*., 2014, 2017; Marin *et al*., 2007; Moraitis *et al*., 2013; Vezzulli *et al*., 2008; Vita & Marin, 2007; Vita *et al*., 2004a), few have considered the potential variation of attributes of the benthic macroinvertebrate assemblages with distance from a tuna farm (Marin *et al*., 2007; Vita & Marin, 2007).

Workers recommend the use of polychaetes (e.g. Mangion *et al*., 2017; Martinez-Garcia *et al*., 2013; Sutherland *et al*., 2007; Tomassetti & Porrello, 2005) and amphipods (e.g. Fernandez-Gonzalez & Sanchez-Jerez, 2011; Mangion *et al*., 2017) as bioindicators for monitoring the influence of fish farm wastes on benthic macroinvertebrate assemblages. More specifically, the polychaete/amphipod (BOPA-Fish farming) ratio (Aguado-Giménez *et al*., 2015) is a useful biotic index that classifies water bodies into ‘High’, ‘Good’, ‘Moderate’, ‘Poor’, or ‘Bad’ Ecological Quality Status (EQS) classes (Dauvin & Ruellet, 2007; Gomez-Gesteira & Dauvin, 2000).

The aim of the present study was to assess for potential variation in attributes of benthic macroinvertebrate assemblages under the influence of tuna penning activities, by

analysing biotic data collected at incremental distances of 0 m, 100 m, 1 km and 2 km from two tuna farms sited circa 1 km off the coast of Malta. The null hypotheses tested are no significant difference in: (a) sediment physico-chemical attributes; (b) abundance of selected indicator families, number of families, Shannon-Wiener diversity, and assemblage composition of polychaete and amphipod assemblages, and (c) the BOPA-Fish farming index, with increasing distance from two tuna farms.

## 5.2 Material and methods

### 5.2.1 Study sites and sampling

The two tuna farms considered in the present study component were located 1 km off the coast of Malta (Figure 5.1) where the seabed consisted of soft sediment. One farm was located off the northeastern coast of Malta where water depth was some 45 – 50 m, while the other farm was located off the southeastern coast of Malta; where water depth was some 42 – 53 m. Each tuna farm had a unique setup and management regime. The northeastern farm (NEF) had eight tuna cages with a maximum total annual capacity of 2500 t while the southeastern farm (southeastern 'Farm 1' [SEF 1]) was smaller and had three tuna cages, with a maximum total annual capacity of 1500 t (ICCAT, 2011). Both farms utilized cages that had a diameter of some 50 m and height of around 25 m.

The sampling design incorporated four sampling plots, which had similar bottom type: (i) farm plot; i.e. the seabed area occupied by the footprint of the tuna cages; (ii) impacted plot; i.e. the seabed area c. 100 m around the tuna farm; (iii) 'Control 1' plot, located c. 1 km from the cages; and (iv) 'Control 2' plot, located c. 2 km away from the cages (Figure 5.1). Three sampling sites were allotted to each plot, since the minimum number of cages at any one of the farms was three. Therefore, the sampling design was replicated at each of the two farms, such that a total of twenty four sampling sites were included in the sampling design. The latitude/longitude coordinates and depth of the sampling sites are given in Table 5.1.

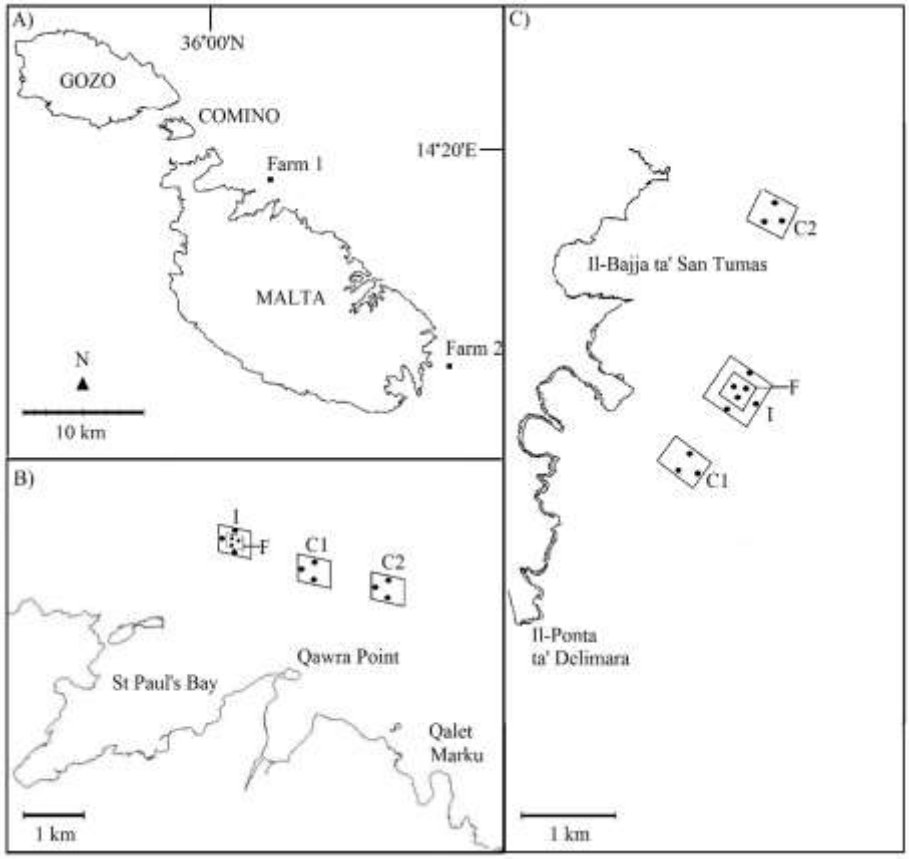


**Figure 5.1** Map of the Maltese Islands showing the locations of: (a) the two tuna farms; (b) the three sampling plots at the northeastern farm; and (c) the three sampling plots at southeastern 'Farm 1'; from where samples for sediment quality and benthic macrofaunal studies were collected. F = farm plot, I = impacted plot, C1 = 'Control 1' plot, C2 = 'Control 2' plot

Sampling of soft sediment assemblages was carried out in November 2003, 2004 and 2005 at the NEF, and in October 2003, 2004 and 2005 at the SEF 1, following initiation of tuna penning activities. Sampling was carried out using a 0.1 $m^2$ van Veen grab. Three replicate grab samples for benthic macrofaunal studies and one grab sample for sediment studies were collected at each of the eighteen sampling sites. The samples collected for faunal studies were live-sieved (0.5 mm mesh) on board the vessel and afterward temporarily preserved in 10 % formalin.

In the laboratory, samples for faunal studies were sorted for polychaetes and amphipods after washing on a 0.5 mm mesh. Macroinvertebrates were identified to the family level and enumerated to obtain estimates of number of families and number of individuals for a given family per grab sample. For sediment physico-chemical studies, sub-samples for the determination of w/w percent feed-fish bone content (FFBC), percent organic carbon content (POCC) and percent organic

**Table 5.1** Latitude/longitude coordinates and depth of the control sites shown in Figure 5.1. The impacted plot at the northeastern farm (NEF) was centered on N35º 58.66'/E14º 25.16', and at southeastern 'Farm 1' (SEF 1) on N35º 50.17'/E14º 35.11'; while samples were also collected from the seabed area directly below the cages.

| NEF | | | |
|---|---|---|---|
| Plot | Site | Latitude/Longitude | Depth (m) |
| Impact | S1 | N35º 58.77/E14º 25.18 | 48 |
| | S2 | N35º 58.63/E14º 25.31 | 48 |
| | S3 | N35º 58.56/E14º 25.18 | 48 |
| 'Control 1' | S1 | N35º 58.51/E14º 26.33 | 52 |
| | S2 | N35º 58.38/E14º 26.47 | 52 |
| | S3 | N35º 58.32/E14º 26.33 | 51 |
| 'Control 2' | S1 | N35º 58.32/E14º 26.72 | 50 |
| | S2 | N35º 58.18/E14º 26.85 | 50 |
| | S3 | N35º 58.12/E14º 26.72 | 48 |
| SEF 1 | | | |
| Plot | Site | Latitude/Longitude | Depth (m) |
| Impact | S1 | N35º 50.61/E14º 35.16 | 50 |
| | S2 | N35º 50.52/E14º 35.25 | 51 |
| | S3 | N35º 50.52/E14º 35.00 | 46 |
| 'Control 1' | S1 | N35º 50.18/E14º 34.79 | 51 |
| | S2 | N35º 50.10/E14º 34.85 | 51 |
| | S3 | N35º 50.11/E14º 34.70 | 46 |
| 'Control 2' | S1 | N35º 51.58/E14º 35.42 | 47 |
| | S2 | N35º 51.50/E14º 35.48 | 47 |
| | S3 | N35º 51.49/E14º 35.37 | 45 |

nitrogen content (PONC) were frozen at -20°C for later analysis, while another sub-sample was oven dried for determination of mean sediment grain size (MSGS).

Analysis of the sediment to determine the FFBC was carried out by sorting the sediment under a dissecting microscope. POCC in the sediment was determined using acid digestion, and PONC in the sediment was determined according to the Kjeldhal method (see Holme & McIntyre, 1984). MSGS of the sediment was determined according to Buchanan (1984).

### 5.2.2 Data analyses

Data on attributes of the polychaete and amphipod assemblages were analysed as follows; macroinvertebrate indicator taxa at family level were selected as the three families in each taxocene that had the highest abundance, hence contributing most to the difference in assemblage composition at incremental distances from the tuna cages. Selection of the following indicator taxa was based on the output from a similarity percentages of species contributions (SIMPER) analysis (Clarke & Warwick, 2001) (see below): Capitellidae, Syllidae and Dorvilleidae (polychaetes), and Urothoidae, Lysianassidae and Photidae (amphipods). Four-factor permutational univariate analysis of variance (PERMANOVA) (Anderson, 2001) was undertaken (with α set at 0.05) using a Euclidean similarity matrix to test the hypothesis of no differences in the abundance of selected indicator taxa, number of taxa and Shannon-Wiener diversity of taxa per faunal group, and in the polychaete/amphipod (BOPA-Fish farming [BOPA-FF]) index as defined by Aguado-Giménez *et al*. (2015) amongst incremental distances from two tuna farms over a three year period. The underlying model had four factors: 'Location' (Lo; 2 levels, NEF and SEF 1, fixed), 'Distance' (Di; 4 levels, Farm, Impact, 'Control 1' and 'Control 2', fixed), 'Time' (Ti; 3 levels, 2003, 2004 and 2005, random), and 'Site' (Si; 3 levels, S1, S2 and S3, random) nested within the 'Lo x Di x Ti' interaction. Separate three-factor univariate PERMANOVA was undertaken using a similar experimental design to test the hypothesis of no differences in the MSGS, POCC and PONC of the sediment. The analysis (with level of significance [α] set at 0.05) was based on a Euclidean similarity matrix, and the design had levels of 'Si' treated as replicates.

Planned contrast tests between different levels representing points at increasing distance from the tuna cages were constructed by combining sum of squares values from three separate PERMANOVA analyses (see Glasby, 1997) to provide for partitioning of the factor 'Di' into three component tests: (i) between the farm lease area, and the average of the impacted, 'Control 1', and 'Control 2' plots ('F-vs-I C1 C2'); (ii) between the impacted plot and the average of the 'Control 1' and 'Control 2' plots ('I-vs-C1 C2'); and (iii) between the 'Control 1' plot and the 'Control 2' plot ('C1-vs-C2'). The numerator/s and denominator/s used to calculate the F ratio for the

individual terms in the three-factor and four-factor PERMANOVAs are respectively given in Tables 5.2 and 5.4.

In the analysis, the main PERMANOVA terms of interest that may be used to assess for a spatial pattern in the influence of tuna penning activities on benthic habitat over time are the 'F-vs-I C1 C2 x Ti', 'I-vs-C1-C2 x Ti', and 'C1-vs-C2 x Ti' interaction terms. The 'Lo x F-vs-I C1 C2 x Ti', 'Lo x I-vs-C1 C2 x Ti', and 'Lo x C1-vs-C2 x Ti' interaction terms indicate variability in the spatial pattern of influence of tuna penning on benthic habitat over time between the two farm locations, while the 'Si(Lo x F-vs-I C1 C2 x Ti)', 'Si(Lo x I-vs-C1 C2 x Ti)', and 'Si(Lo x C1-vs-C2 x Ti)' interaction terms indicate temporal variability at the smallest spatial scale (of a few meters).

To test the hypothesis of no difference in family abundance of polychaete and amphipod fauna over time with increasing distance from a tuna farm, four-factor permutational multivariate ANOVA (PERMANOVA) (Anderson, 2001; McArdle & Anderson, 2001) was carried out (α set at 0.05) using a Bray Curtis similarity matrix calculated from the family abundance data that were fourth-root transformed to downweigh the highly abundant taxa (Clarke & Warwick, 2001). A permutational multivariate dispersion test (PERMDISP) (Anderson, 2004, 2006) was then used to calculate differences (α set at 0.05) in within-group dispersion using the sample distance to the centroid of each of the different factors. In both PERMANOVA and PERMDISP tests, a total of 9999 unrestricted permutations of raw data were used, with α set at 0.05. When the number of possible unique permutations was not sufficient to get a reliable permutation test, the Monte Carlo test was used instead (Anderson & Robinson, 2003). Principal coordinate analysis (PCO) (Anderson, 2003) was run and the results were plotted to show differences in the polychaete and amphipod assemblage composition with increasing distance from the tuna farms over time. The three most important taxa contributing to the similarity of samples within plots, and to the dissimilarity of samples between incremental distances from the tuna pens for each of the two farms, and between the two farms per plot, were identified using the similarity percentages of species contributions (SIMPER) method (Clarke & Warwick, 2001). *A posteriori* univariate PERMANOVA was run (with α set at 0.05) on the taxa that contributed most to the dissimilarity between samples collected at increasing

distances from the tuna cages at each of the two farms using the term 'Si(Ti x Di)'. To test for significant differences in sediment physico-chemical variables, similar multivariate analyses were run, based on the three-factor model and using a D1 Euclidean similarity matrix calculated from environmental data that was normalised to homogenize the different units, i.e. mm for MSGS, and % for POCC and PONC (Clarke & Warwick, 2001). To determine which sediment physico-chemical attribute or combination thereof best explained the observed variation in polychaete and amphipod assemblages recorded at incremental distances from the tuna pens per farm, the BEST routine of the biota and/or environment matching (BIOENV) analysis (Clarke & Gorley, 2006) was carried out, using the Spearman rank correlation method and D1 Euclidean similarity measure (Clarke & Warwick, 2001). All the analyses were undertaken using PRIMER v.7.0.11 (PRIMER software; Clarke & Gorley, 2006) and the PERMANOVA+ v.1.0 add-on package (Anderson *et al*., 2008).

## 5.3 Results

### 5.3.1 Univariate data analyses

#### 5.3.1(i) Sediment physico-chemical attributes

Values of mean % FFBC of the sediment recorded below tuna cages at the SEF 1 were high compared to those recorded at the NEF, and decreased overall during the study period (from 1.594 % $\pm$ 2.656 % to 0.032 % $\pm$ 0.045 %, and from 1.041 % $\pm$ 0.600 % to 0.146 % $\pm$ 0.109 %, respectively). Univariate PERMANOVA indicated no significant difference in MSGS, POCC and PONC for the 'Ti x F-vs-I C1 C2', 'Ti x I-vs-C1 C2', and 'Ti x C1-vs-C2' interaction terms, while 'Lo x I-vs-C1 C2 x Ti' was significant for POCC ($p < 0.01$) and PONC ($p < 0.05$) (Table 5.2). Pair-wise tests showed that PONC at the impacted plot was significantly higher ($p < 0.05$) in 2003, significantly lower ($p < 0.05$) in 2004 at the NEF, and significantly higher ($p < 0.05$) in 2004 at the SEF 1, compared to the control plots. Pair-wise tests also showed that POCC and PONC at the impacted plot were significantly lower at the NEF compared to the SEF 1 respectively in 2003 ($p < 0.05$) and 2004 ($p < 0.01$) (Figure 5.2, Table 5.2).

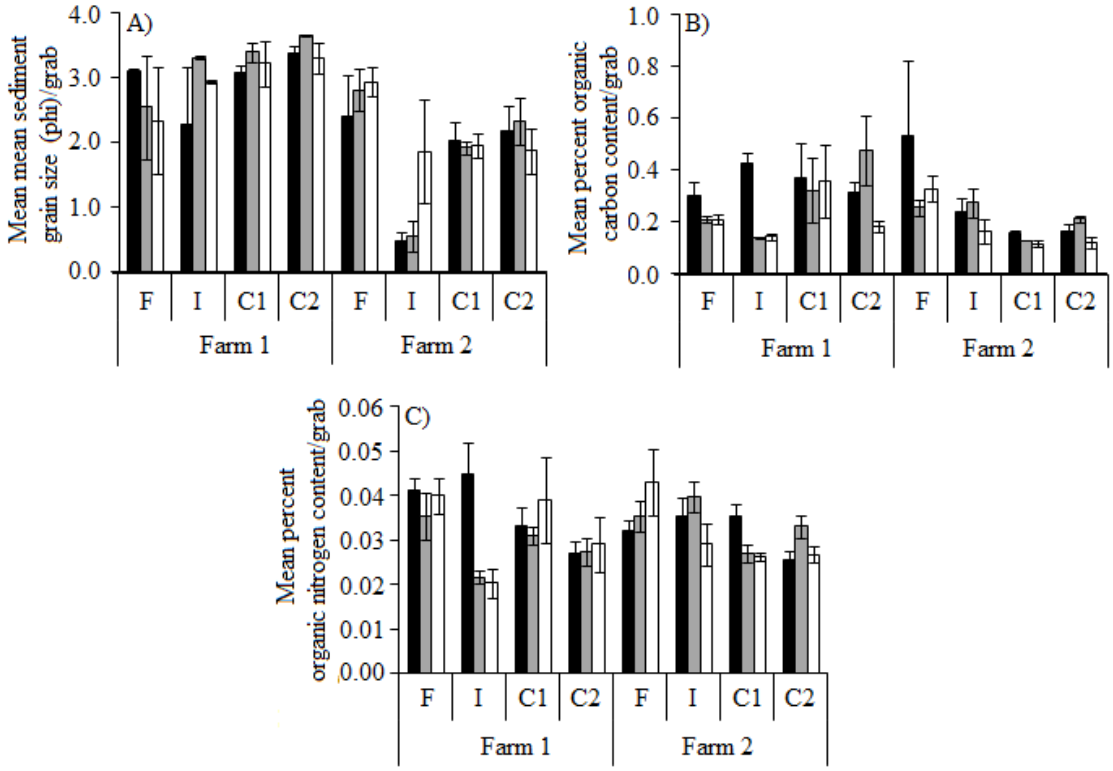


**Figure 5.2** Mean values (±SE) per grab of: (a) mean sediment grain size (phi), (b) percent organic carbon content, and (c) percent organic nitrogen content, recorded at the northeastern farm (NEF) and southeastern 'Farm 1' (SEF 1) farm (F), impacted (I), 'Control 1' (C1) and 'Control 2' (C2) plots in the years 2003 (black bars), 2004 (gray bars) and 2005 (white bars).

### 5.3.1(ii) Macroinvertebrate assemblages

A total of 9,342 individuals from 23 polychaete families, and 2,093 individuals from 22 amphipod families were collected from the NEF sampling plots. SIMPER analysis showed that the three most important taxa (in terms of abundance) contributing to the similarity of the amphipod assemblages at the NEF made up c. 19% of the total amphipod abundance within the farm plot, while taxa showed higher dominance at the impacted and control plots (c. 49-52 %) (Table 5.3). The three most important polychaete taxa made up half (c. 42-53 %) the total polychaete abundance at the four NEF plots. The polychaete and amphipod assemblages differed by more than half (c. 60 % and c. 70 %, respectively) between the farm plot, and the impacted and control plots. The polychaete taxa that contributed most to this dissimilarity were Capitellidae, which were more abundant at the farm plot, and Maldanidae and Sabellidae, which were less abundant at the farm plot, compared to the impacted and control plots. The amphipod taxa (i.e. Phoxocephalidae, Urothoidae and Lysianassidae) that contributed

**Table 5.2** Results of univariate three-factor PERMANOVA for mean sediment grain size (phi) (MSGS), percent organic carbon content (POCC) and percent organic nitrogen content (PONC), with planned contrast tests for the factor 'Distance'; the F-ratio numerator/s and denominator/s are indicated. RES = Residual

| Source of Variation | df | MSGS | POCC | PONC | F-ratio Numerator | F-ratio Denominator |
|---|---|---|---|---|---|---|
| Location = Lo | 1 | * | ns | ns | Lo and Lo x Di x Ti | Lo x Di and Lo x Ti |
| Distance = Di | 3 | ns | ns | ns | Di and Lo x Di x Ti | Lo x Di and Ti x Di |
| F-vs-I C1 C2 | 1 | ns | ns | ns | F-vs-I C1 C2 and Lo x F-vs-I C1 C2 x Ti | Lo x F-vs-I C1 C2 and Ti x F-vs-I C1 C2 |
| I-vs-C1 C2 | 1 | ns | ns | ns | I-vs-C1 C2 and Lo x I-vs-C1 C2 x Ti | Lo x I-vs-C1 C2 and Ti x I-vs-C1 C2 |
| C1-vs-C2 | 1 | ns | ns | ns | C1-vs-C2 and Lo x C1-vs-C2 x Ti | Lo x C1-vs-C2 and Ti x C1-vs-C4 |
| Time = Ti | 2 | ns | * | ns | Ti and Lo x Di x Ti | Lo x Ti and Ti x Di |
| Lo x Di | 3 | * | ns | ns | Lo x Di | Lo x Di x Ti |
| Lo x F-vs-I C1 C2 | 1 | ns | ns | ns | Lo x F-vs-I C1 C2 | Lo x F-vs-I C1 C2 x Ti |
| Lo x I-vs-C1 C2 | 1 | ns | ns | ns | Lo x I-vs-C1 C2 | Lo x I-vs-C1 C2 x Ti |
| Lo x C1-vs-C2 | 1 | ns | ns | ns | Lo x C1-vs-C2 | Lo x C1-vs-C2 x Ti |
| Lo x Ti | 2 | ns | ns | ns | Lo x Ti | Lo x Di x Ti |
| Ti x Di | 6 | ns | ns | ns | Ti x Di | Lo x Di x Ti |
| Ti x F-vs-I C1 C2 | 2 | ns | ns | ns | Ti x F-vs-I C1 C2 | Lo x F-vs-I C1 C2 x Ti |
| Ti x I-vs-C1 C2 | 2 | ns | ns | ns | Ti x I-vs-C1 C2 | Lo x I-vs-C1 C2 x Ti |
| Ti x C1-vs-C2 | 2 | ns | ns | ns | Ti x C1-vs-C2 | Lo x C1-vs-C2 x Ti |
| Lo x Di x Ti | 6 | ns | ns | ns | Lo x Di x Ti | RES(Lo x Di x Ti) |
| Lo x F-vs-I C1 C2 x Ti | 2 | ns | ns | ns | Lo x F-vs-I C1 C2 x Ti | RES(Lo x F-vs-I C1 C2 x Ti) |
| Lo x I-vs-C1 C2 x Ti | 2 | ns | ** | * | Lo x I-vs-C1 C2 x Ti | RES(Lo x I-vs-C1 C2 x Ti) |
| Lo x C1-vs-C2 x Ti | 2 | ns | ns | ns | Lo x C1-vs-C2 x Ti | RES(Lo x C1-vs-C2 x Ti) |
| RES(Lo x Di x Ti) | 48 | | | | | |
| RES(Lo x F-vs-I C1 C2 x Ti) | 12 | | | | | |
| RES(Lo x I-vs-C1 C2 x Ti) | 12 | | | | | |
| RES(Lo x C1-vs-C2 x Ti) | 24 | | | | | |

**Table 5.2** Continued

| Source of Variation | | df | MSGS | POCC | PONC |
|---|---|---|---|---|---|
| Total | | 71 | | | |
| *Pair-wise tests -: 'Lo x I-vs-C1 C2 x Ti'* | | | | | |
| I-vs- C1 C2 | 'Farm 1' | 2003 | - | ns | > * |
| | | 2004 | - | ns | < * |
| | | 2005 | - | ns | ns |
| | 'Farm 2' | 2003 | - | ns | ns |
| | | 2004 | - | ns | > * |
| | | 2005 | - | ns | ns |
| 'Farm 1' vs 'Farm 2' | Impact | 2003 | - | < * | ns |
| | | 2004 | - | ns | < ** |
| | | 2005 | - | ns | ns |

most to this dissimilarity were less abundant at the farm plot compared to the impacted and control plots. The polychaete and amphipod taxa differed by half between the impacted plot and the control plots (c. 49 % and c. 50 %, respectively), and between the two control plots (c. 47%) (Table 5.3).

A total of 11,255 individuals from 27 polychaete families and 2,607 individuals from 23 amphipod families were collected from the SEF 1 plots. SIMPER analysis showed high taxon dominance (c. 58-67 %) for polychaete assemblages at each of the SEF 1 plots, and low taxon dominance (c. 22-27 %) for amphipod assemblages at the farm and impacted plots of the SEF 1 (Table 5.3). The top three amphipod taxa contributed to circa half the total amphipod abundance (c. 54 %) at the SEF 1 control plots. The amphipod assemblages differed by c. 72 % between the farm plot, and the impacted and control plots, and by c. 66 % between the impacted plot and the control plots. The amphipod taxa that contributed most to the dissimilarity at the farm plot were Urothoidae, Lysianassidae and Ampeliscidae, which were less abundant at the farm plot compared to the impacted and control plots. The taxa that contributed most to the dissimilarity at the impacted plot were Urothoidae, Photidae and Lysianassidae, which were more abundant at the impacted plot compared to the control plots (Table 5.3). Polychaete family abundance differed by less than half (c. 35-46%) at increasing distances from the SEF 1 tuna cages (Table 5.3).

SIMPER analysis indicated high dissimilarity in amphipod family abundance between the NEF and the SEF 1 at farm (c. 79%) and impacted (c. 68%) plots, and lower dissimilarity (c. 51-52%) at control plots, while polychaete family abundances differed by c. 54-60% between the two study areas. In general, polychaete and amphipod abundance was lower at the NEF compared to the SEF 1 (Table 5.3).

Univariate PERMANOVA indicated no significant difference in the abundance of polychaetes and amphipods for the interaction terms ‘Ti x F-vs-I C1 C2’, ‘Ti x I-vs-C1 C2’, and ‘Ti x C1-vs-C2’ (Table 5.4). PERMANOVA indicated significant differences for ‘Lo x F-vs-I C1 C2 x Ti’ in the abundance of Capitellidae ($p < 0.05$); for ‘Lo x I-vs-C1 C2 x Ti’ in abundance of Photidae ($p < 0.001$); and for ‘Lo x C1- vs-C2 x Ti’ in abundance of Syllidae ($p < 0.01$), Urothoidae ($p < 0.05$), and Photidae

**Table 5.3** Results of SIMPER analysis calculated from a Bray Curtis similarity matrix of fourth-root transformed family abundance data, showing the top three polychaete and amphipod families (in terms of abundance) that contributed to the similarity of samples within plots, and to the dissimilarity of samples between incremental distances from the tuna pens per farm and between the two farms per plot. Avg Sim (%) = average similarity (%), Cum (%) = cumulative contribution (%), Avg Diss (%) = average dissimilarity (%), Avg Abund = average abundance, Contrib (%) = contribution (%), NEF = northeastern farm, SEF 1 = southeastern 'Farm 1'

| | | Polychaetes | | | Amphipods | | |
|---|---|---|---|---|---|---|---|
| | Distance | Avg Sim (%) | Family | Cum (%) | Avg Sim (%) | Family | Cum (%) |
| NEF | Farm | 42.05 | Paraonidae | 34.54 | 19.00 | Lysianassidae | 31.44 |
| | | | Capitellidae | 63.69 | | Urothoidae | 57.05 |
| | | | Opheliidae | 72.71 | | Maeridae | 74.31 |
| | Impact | 48.93 | Paraonidae | 23.19 | 43.51 | Phoxocephalidae | 30.03 |
| | | | Sabellidae | 34.91 | | Lysianassidae | 59.90 |
| | | | Maldanidae | 46.18 | | Urothoidae | 81.11 |
| | 'Control 1' | 53.12 | Paraonidae | 22.61 | 55.30 | Phoxocephalidae | 30.25 |
| | | | Maldanidae | 34.15 | | Lysianassidae | 57.17 |
| | | | Opheliidae | 44.81 | | Urothoidae | 70.48 |
| | 'Control 2' | 52.90 | Paraonidae | 23.76 | 52.33 | Urothoidae | 26.50 |
| | | | Maldanidae | 35.12 | | Lysianassidae | 52.27 |
| | | | Sabellidae | 45.29 | | Phoxocephalidae | 76.25 |
| SEF 1 | Farm | 57.85 | Capitellidae | 18.03 | 22.18 | Urothoidae | 42.50 |
| | | | Cirratulidae | 30.94 | | Phoxocephalidae | 57.16 |
| | | | Paraonidae | 43.72 | | Maeridae | 71.33 |
| | Impact | 62.90 | Paraonidae | 11.12 | 27.43 | Maeridae | 22.77 |
| | | | Capitellidae | 19.11 | | Lysianassidae | 43.36 |
| | | | Glyceridae | 26.70 | | Ampeliscidae | 58.62 |
| | 'Control 1' | 64.55 | Maldanidae | 16.18 | 53.74 | Ampeliscidae | 23.10 |
| | | | Paraonidae | 30.88 | | Lysianassidae | 44.76 |
| | | | Glyceridae | 45.01 | | Urothoidae | 63.26 |

**Table 5.3** Continued

| | | | Polychaetes | | | | Amphipods | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Distance | Avg Sim (%) | Family | Cum (%) | | Avg Sim (%) | Family | Cum (%) | | |
| SEF 1 | 'Control 2' | 66.52 | Maldanidae | 14.57 | | 54.40 | Urothoidae | 28.08 | | |
| | | | Glyceridae | 28.85 | | | Ampeliscidae | 50.00 | | |
| | | | Paraonidae | 45.90 | | | Photidae | 69.27 | | |
| | | Avg | | Avg Abund | | | Avg | | Avg Abund | | Avg |
| | Groups | Diss (%) | Family | x | y | Contrib% | Diss (%) | Family | x | y | Diss (%) |
| NEF | x = F | 59.83 | Capitellidae | 2.26 | 0.90 | 13.90 | 70.28 | Phoxocephalidae | 0.47 | 1.33 | 15.61 |
| | y = I C1 C2 | | Maldanidae | 0.29 | 1.17 | 7.46 | | Urothoidae | 0.59 | 1.16 | 14.06 |
| | | | Sabellidae | 0.51 | 1.05 | 6.37 | | Lysianassidae | 0.66 | 1.23 | 13.68 |
| | x = I | 49.41 | Nereididae | 0.99 | 0.86 | 6.49 | 49.85 | Urothoidae | 1.17 | 1.15 | 13.78 |
| | y = C1 C2 | | Opheliidae | 0.88 | 0.91 | 6.30 | | Phoxocephalidae | 0.28 | 0.78 | 10.86 |
| | | | Capitellidae | 1.01 | 0.85 | 6.26 | | Ampeliscidae | 0.54 | 0.81 | 10.37 |
| | x = C1 | 47.46 | Opheliidae | 1.20 | 0.61 | 7.55 | 46.59 | Photidae | 0.91 | 0.65 | 12.80 |
| | y = C2 | | Hesionidae | 0.99 | 0.76 | 6.96 | | Urothoidae | 1.01 | 1.30 | 12.16 |
| | | | Maldanidae | 1.25 | 1.01 | 6.63 | | Ampeliscidae | 0.85 | 0.76 | 10.97 |
| | x = F | 44.74 | Capitellidae | 2.53 | 1.00 | 10.63 | 72.37 | Urothoidae | 0.92 | 1.04 | 11.73 |
| | y = I C1 C2 | | Dorvilleidae | 0.86 | 0.60 | 5.65 | | Lysianassidae | 0.66 | 1.01 | 11.66 |
| | | | Syllidae | 0.58 | 1.02 | 5.57 | | Ampeliscidae | 0.27 | 0.97 | 10.93 |
| SEF 1 | x = I | 43.38 | Dorvilleidae | 1.41 | 0.20 | 7.69 | 66.25 | Urothoidae | 1.26 | 0.58 | 11.37 |
| | y = C1 C2 | | Nereididae | 1.34 | 0.30 | 6.96 | | Photidae | 1.08 | 0.25 | 11.29 |
| | | | Syllidae | 1.48 | 0.79 | 6.45 | | Lysianassidae | 1.12 | 0.79 | 10.14 |
| | x = C1 | 35.06 | Syllidae | 0.81 | 0.77 | 7.07 | 46.84 | Lysianassidae | 1.33 | 0.92 | 11.66 |
| | y = C2 | | Eunicidae | 0.94 | 0.57 | 6.53 | | Photidae | 0.95 | 1.21 | 10.29 |
| | | | Orbiniidae | 1.03 | 0.93 | 6.27 | | Urothoidae | 1.18 | 1.34 | 8.81 |

($p < 0.05$). Pair-wise tests showed a significantly higher abundance of Capitellidae at the farm plot compared to the impacted and control plots at the NEF ($p < 0.001$) and the SEF 1 ($p_{2003} < 0.01$, $p_{2005} < 0.0001$) in 2003 and 2005, and a significantly higher ($p < 0.001$) abundance of Syllidae at the 'Control 1' plot compared to 'Control 2' plot at the SEF 1 in 2003 (Figure 5.3). The abundance of Capitellidae below tuna cages was significantly higher ($p < 0.05$) at the SEF 1 compared to the NEF in 2005. The results of pair-wise tests showed a significantly lower ($p < 0.05$) abundance of Photidae at the impacted plot compared to the control plots at the NEF in 2003 and 2005, and at the SEF 1 in 2003, 2004 and 2005; and a significantly lower ($p < 0.05$) abundance of Urothoidae at the 'Control 1' plot compared to the 'Control 2' plot at the SEF 1 in 2005 (Figure 5.3).

PERMANOVA also indicated significant differences for 'Si(Lo x F-vs-I C1 C2 x Ti)' in the abundance of Lysianassidae ($p < 0.05$) and Photidae ($p < 0.05$); for 'Si(Lo x I-vs-C1 C2 x Ti)' in the abundance of Capitellidae ($p < 0.001$), Syllidae ($p < 0.01$), Urothoidae ($p < 0.001$), and Lysianassidae ($p < 0.01$); and for 'Si(Lo x C1-vs-C2 x Ti)' in the abundance of Photidae ($p < 0.05$) (Table 5.4).

PERMANOVA indicated no significant difference for 'Ti x F-vs-I C1 C2', 'Ti x I-vs-C1 C2', and 'Ti x C1-vs-C2' in the number of polychaete families, and number and Shannon-Wiener diversity of amphipods, while a significant difference was indicated for 'Ti x I-vs-C1 C2' ($p < 0.05$) in the Shannon-Wiener diversity of polychaetes (Table 5.4). PERMANOVA also indicated a significant difference for 'Lo x F-vs-I C1 C2 x Ti' in the Shannon-Wiener diversity of polychaetes ($p < 0.01$), and number of families ($p < 0.01$) and Shannon-Wiener diversity ($p < 0.001$) of amphipod families; for 'Lo x I-vs-C1 C2 x Ti' in the number ($p < 0.05$) and Shannon-Wiener diversity ($p < 0.01$) of amphipod families; and for 'Lo x C1-vs-C2 x Ti' in the number of polychaete families ($p < 0.01$), and for number ($p < 0.01$) and Shannon-Wiener diversity ($p < 0.05$) of amphipod families (Table 5.4).

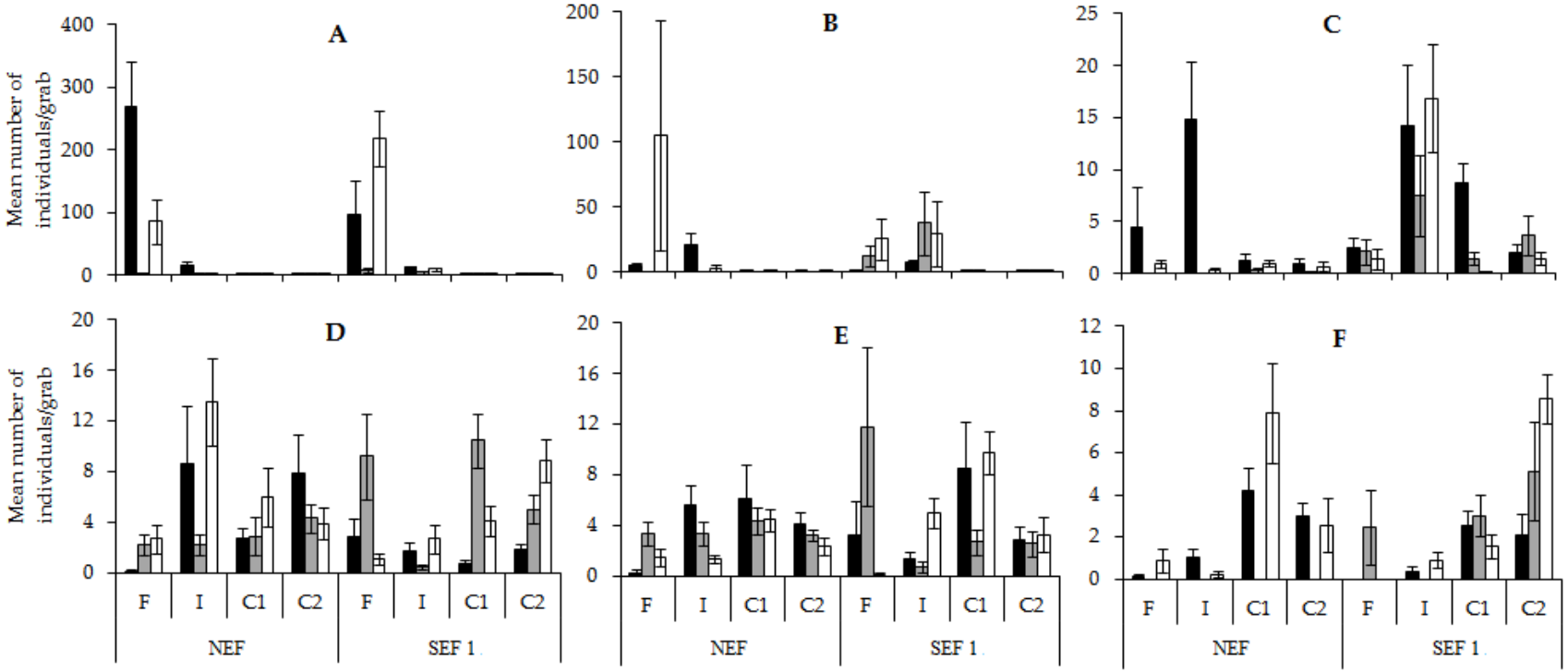


**Figure 5.3** Mean values (± SE) per grab of number of individuals of: (i) polychaete indicator taxa (a) Capitellidae, (b) Syllidae, and (c) Dorvilleidae; and (ii) amphipod indicator taxa (d) Urothoidae, (e) Lysianassidae, and (f) Photidae; recorded at the northeastern farm (NEF) and southeastern 'Farm 1' (SEF 1) farm (F), impacted (I), 'Control 1' (C1) and 'Control 2' (C2) plots in the years 2003 (black bars), 2004 (gray bars), and 2005 (white bars).

**Table 5.4** Results of four-factor univariate PERMANOVA for number of individuals (NI) of selected indicator taxa Capitellidae (1), Syllidae (2) and Dorvilleidae (3) (polychaetes), and Urothoidae (1), Lysianassidae (2) and Photidae (3) (amphipods); number of families (NFa) and Shannon-Wiener diversity (ShW) of polychaetes and amphipods; and the polychaete/amphipod (BOPA-Fish farming; BOPA-FF) index, with planned contrast tests for the factor 'Distance'. The F-ratio numerator/s and denominator/s are indicated. Df = degrees of freedom, RES = Residual, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1'

| | | Polychaetes | | | | | Amphipods | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | df | NI 1 | NI 2 | NI 3 | NFa | ShW | NI 1 | NI 2 | NI 3 | NFa | ShW | BOPA-FF |
| Location = Lo | 1 | ns | ns | ns | ** | ** | ns | ns | ns | Ns | ns | ns |
| Distance = Di | 3 | * | ns | * | ns | * | ns | ns | ns | ns | ** | ns |
| F-vs-I C1 C2 | 1 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| I-vs-C1 C2 | 1 | ns | ns | ns | ns | ns | ns | ns | * | ns | ns | ns |
| C1-vs-C2 | 1 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Time = Ti | 2 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Lo x Di | 3 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Lo x F-vs-I C1 C2 | 1 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Lo x I-vs-C1 C2 | 1 | ns | ns | ns | ns | * | ns | ns | ns | ns | ns | ns |
| Lo x C1-vs-C2 | 1 | * | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Lo x Ti | 2 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Ti x Di | 6 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Ti x F-vs-I C1 C2 | 2 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Ti x I-vs-C1 C2 | 2 | ns | ns | ns | ns | * | ns | ns | ns | ns | ns | ns |
| Ti x C1-vs-C2 | 2 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Lo x Di x Ti | 6 | *** | ns | ns | * | ** | ns | ns | * | **** | **** | ns |
| Lo x F-vs-I C1 C2 x Ti | 2 | * | ns | ns | ns | ** | ns | ns | ns | ** | *** | ns |
| Lo x I-vs-C1 C2 x Ti | 2 | ns | ns | ns | ns | ns | ns | ns | *** | * | ** | ns |
| Lo x C1-vs-C2 x Ti | 2 | ns | ns | ** | ** | ns | * | ns | * | ** | * | ns |

**Table 5.4** Continued

| | | | Polychaetes | | | | | Amphipods | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | | df | NI 1 | NI 2 | NI 3 | NFa | ShW | NI 1 | NI 2 | NI 3 | NFa | ShW | BOPA-FF |
| Site(Lo x Di x Ti) = Si(Lo x Di x Ti) | | 48 | ns | ns | **** | ** | ** | ** | ** | ** | * | * | **** |
| Si(Lo x F-vs-I C1 C2 x Ti) | | 12 | ns | ns | ns | ** | ns | ns | * | * | ns | ns | *** |
| Si(Lo x I-vs-C1 C2 x Ti) | | 12 | *** | ns | ** | ns | ns | *** | ** | ns | ns | ns | ** |
| Si(Lo x C1-vs-C2 x Ti) | | 24 | ns | ns | ns | ns | **** | ns | ns | * | ns | * | ns |
| RES: Si(Lo x Di x Ti) | | 144 | | | | | | | | | | | |
| RES: Si(Lo x F-vs-I C1 C2 x Ti) | | 36 | | | | | | | | | | | |
| RES: Si(Lo x I-vs-C1 C2 x Ti) | | 36 | | | | | | | | | | | |
| RES: Si(Lo x C1-vs-C2 x Ti) | | 72 | | | | | | | | | | | |
| Total | | 215 | | | | | | | | | | | |
| *Pair-wise tests -: 'Lo x Di x Ti'* | | | | | | | | | | | | | |
| NEF | F-vs-I C1 C2 | 2003 | > *** | - | - | - | < ** | - | - | - | < ** | < ** | - |
| | | 2004 | ns | - | - | - | ns | - | - | - | ns | ns | - |
| | | 2005 | > *** | - | - | - | < ** | - | - | - | < * | ns | - |
| | I-vs-C1 C2 | 2003 | - | - | - | - | - | - | - | < * | ns | < * | - |
| | | 2004 | - | - | - | - | - | - | - | ns | ns | ns | - |
| | | 2005 | - | - | - | - | - | - | - | < * | ns | ns | - |
| | C1-vs-C2 | 2003 | - | - | ns | ns | - | ns | - | ns | ns | ns | - |
| | | 2004 | - | - | ns | < ** | - | ns | - | ns | < ** | < * | - |
| | | 2005 | - | - | ns | > ** | - | ns | - | ns | < * | > * | - |
| SEF 1 | F-vs-I C1 C2 | 2003 | > ** | - | - | - | ns | - | - | - | ns | ns | - |
| | | 2004 | ns | - | - | - | ns | - | - | - | ns | ns | - |
| | | 2005 | > *** | - | - | - | < ** | - | - | - | < ** | < ** | - |

**Table 5.4** Continued

| | | | Polychaetes | | | | | Amphipods | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Source of Variation | | df | NI 1 | NI 2 | NI 3 | NFa | ShW | NI 1 | NI 2 | NI 3 | NFa | ShW | BOPA-FF |
| *Pair-wise tests -: 'Lo x Di x Ti'* | | | | | | | | | | | | | |
| SEF 1 | I-vs-C1 C2 | 2003 | - | - | - | - | - | - | - | < * | < ** | < ** | - |
| | | 2004 | - | - | - | - | - | - | - | < * | < ** | < ** | - |
| | | 2005 | - | - | - | - | - | - | - | < * | > ** | > ** | - |
| | C1-vs-C2 | 2003 | - | - | > * | ns | - | ns | - | - | > * | ns | - |
| | | 2004 | - | - | ns | ns | - | > * | - | - | ns | ns | - |
| | | 2005 | - | - | ns | < * | - | < * | - | - | ns | ns | - |
| NEF vs SEF 1 | Farm | 2003 | ns | - | - | - | < ** | - | - | - | < ** | < ** | - |
| | | 2004 | ns | - | - | - | ns | - | - | - | ns | ns | - |
| | | 2005 | < * | - | - | - | ns | - | - | - | > ** | > ** | - |
| | Impact | 2003 | - | - | - | - | - | - | - | ns | ns | ns | - |
| | | 2004 | - | - | - | - | - | - | - | ns | ns | > * | - |
| | | 2005 | - | - | - | - | - | - | - | ns | < * | > ** | - |
| | 'Control 1' | 2003 | - | - | < ** | ns | - | > * | - | ns | ns | ns | - |
| | | 2004 | - | - | ns | < ** | - | > * | - | < ** | < ** | < ** | - |
| | | 2005 | - | - | > * | ns | - | ns | - | > * | < * | < ** | - |
| | 'Control 2' | 2003 | - | - | ns | ns | - | ns | - | ns | ns | ns | - |
| | | 2004 | - | - | ns | ns | - | ns | - | ns | ns | ns | - |
| | | 2005 | - | - | ns | < ** | - | < * | - | > ** | ns | ns | - |

**Table 5.4** Continued

| Source of Variation | F-ratio Numerator | F-ratio Denominator |
|---|---|---|
| Location = Lo | Lo and Lo x Di x Ti | Lo x Di and Lo x Ti |
| Distance = Di | Di and Lo x Di x Ti | Lo x Di and Ti x Di |
| F-vs-I C1 C2 | F-vs-I C1 C2 and Lo x F-vs-I C1 C2 x Ti | Lo x F-vs-I C1 C2 and Ti x F-vs-I C1 C2 |
| I-vs-C1 C2 | I-vs-C1 C2 and Lo x I-vs-C1 C2 x Ti | Lo x I-vs-C1 C2 and Ti x I-vs-C1 C2 |
| C1-vs-C2 | C1-vs-C2 and Lo x C1-vs-C2 x Ti | Lo x C1-vs-C2 and Ti x C1-vs-C4 |
| Time = Ti | Ti and Lo x Di x Ti | Lo x Ti and Ti x Di |
| Lo x Di | Lo x Di | Lo x Di x Ti |
| Lo x F-vs-I C1 C2 | Lo x F-vs-I C1 C2 | Lo x F-vs-I C1 C2 x Ti |
| Lo x I-vs-C1 C2 | Lo x I-vs-C1 C2 | Lo x I-vs-C1 C2 x Ti |
| Lo x C1-vs-C2 | Lo x C1-vs-C2 | Lo x C1-vs-C2 x Ti |
| Lo x Ti | Lo x Ti | Lo x Di x Ti |
| Ti x Di | Ti x Di | Lo x Di x Ti |
| Ti x F-vs-I C1 C2 | Ti x F-vs-I C1 C2 | Lo x F-vs-I C1 C2 x Ti |
| Ti x I-vs-C1 C2 | Ti x I-vs-C1 C2 | Lo x I-vs-C1 C2 x Ti |
| Ti x C1-vs-C2 | Ti x C1-vs-C2 | Lo x C1-vs-C2 x Ti |
| Lo x Di x Ti | Lo x Di x Ti | Site(Lo x Di x Ti) |
| Lo x F-vs-I C1 C2 x Ti | Lo x F-vs-I C1 C2 x Ti | Site(Lo x F-vs-I C1 C2 x Ti) |
| Lo x I-vs-C1 C2 x Ti | Lo x I-vs-C1 C2 x Ti | Site(Lo x I-vs-C1 C2 x Ti) |
| Lo x C1-vs-C2 x Ti | Lo x C1-vs-C2 x Ti | Site(Lo x C1-vs-C2 x Ti) |
| Site(Lo x Di x Ti) = Si(Lo x Di x Ti) | Si(Lo x Di x Ti) | RES: Si(Lo x Di x Ti) |
| Si(Lo x F-vs-I C1 C2 x Ti) | Si(Lo x F-vs-I C1 C2 x Ti) | RES: Si(Lo x F-vs-I C1 C2 x Ti) |
| Si(Lo x I-vs-C1 C2 x Ti) | Si(Lo x I-vs-C1 C2 x Ti) | RES: Si(Lo x I-vs-C1 C2 x Ti) |
| Si(Lo x C1-vs-C2 x Ti) | Si(Lo x C1-vs-C2 x Ti) | RES: Si(Lo x C1-vs-C2 x Ti) |
| RES: Si(Lo x Di x Ti) | | |
| RES: Si(Lo x F-vs-I C1 C2 x Ti) | | |
| RES: Si(Lo x I-vs-C1 C2 x Ti) | | |
| RES: Si(Lo x C1-vs-C2 x Ti) | | |

Pair-wise tests showed significantly lower Shannon-Wiener diversity of polychaetes, and number and Shannon-Weiner diversity of amphipod families at the farm plot compared to the impacted and control plots, at the NEF and the SEF 1 in 2003 ($p < 0.01$); and significantly lower Shannon-Weiner diversity of polychaetes ($p < 0.01$) and number of amphipod families ($p < 0.05$) at the farm plot compared to the impacted and control plots at the NEF in 2005 (Figure 5.4, Table 5.4). The Shannon-Wiener diversity of amphipods at the NEF in 2003 ($p < 0.05$), and number and Shannon-Wiener diversity of amphipod families at the SEF 1 in 2003 and 2004 ($p < 0.01$), were significantly lower at the impacted plot compared to the control plots (Figure 5.4, Table 5.4).

The number and Shannon-Wiener diversity of amphipod families at the SEF 1 were significantly higher ($p < 0.01$) at the impacted plot compared to the control plots in 2005 (Figure 5.4, Table 5.4). Pair-wise tests also showed significantly lower ($p < 0.01$) Shannon-Wiener diversity of polychaetes and amphipods, and number of amphipod families at the farm plot in 2003; and significantly higher ($p < 0.05$) number and Shannon-Wiener diversity of amphipod families at the farm plot in 2005; at the NEF compared to the SEF 1 (Figure 5.4, Table 5.4).

PERMANOVA also indicated significant differences for 'Si(Lo x F-vs-I C1 C2 x Ti)'in the number of polychaete families, and for 'Si(Lo x C1-vs-C2 x Ti)' in Shannon-Wiener diversity of polychaetes ($p < 0.0001$) and amphipods ($p < 0.05$) (Table 5.4).

Mean values of BOPA-FF at the NEF indicated 'Bad' EQS at the farm plot, and 'Good' EQS at the impacted and control plots, in 2003; 'Poor' EQS at the farm plot and 'Good' EQS at the impacted and control plots, in 2005; and 'Good' EQS at the farm and impacted plots, and 'High' EQS at control plots, in 2004 (Figure 5.4). At the SEF 1, BOPA-FF indicated 'Moderate' EQS at the farm plot, 'Poor' EQS at the impacted plot, and 'Good' EQS at the control plots, in 2003; 'Poor' EQS at the impacted plot and 'Good' EQS at the farm and control plots, in 2004; and 'Bad' EQS at the farm plot, and 'Good' EQS at the impacted and control plots, in 2005 (Figure 5.4). PERMANOVA indicated no significant difference in BOPA-FF for 'Ti x F-vs-I

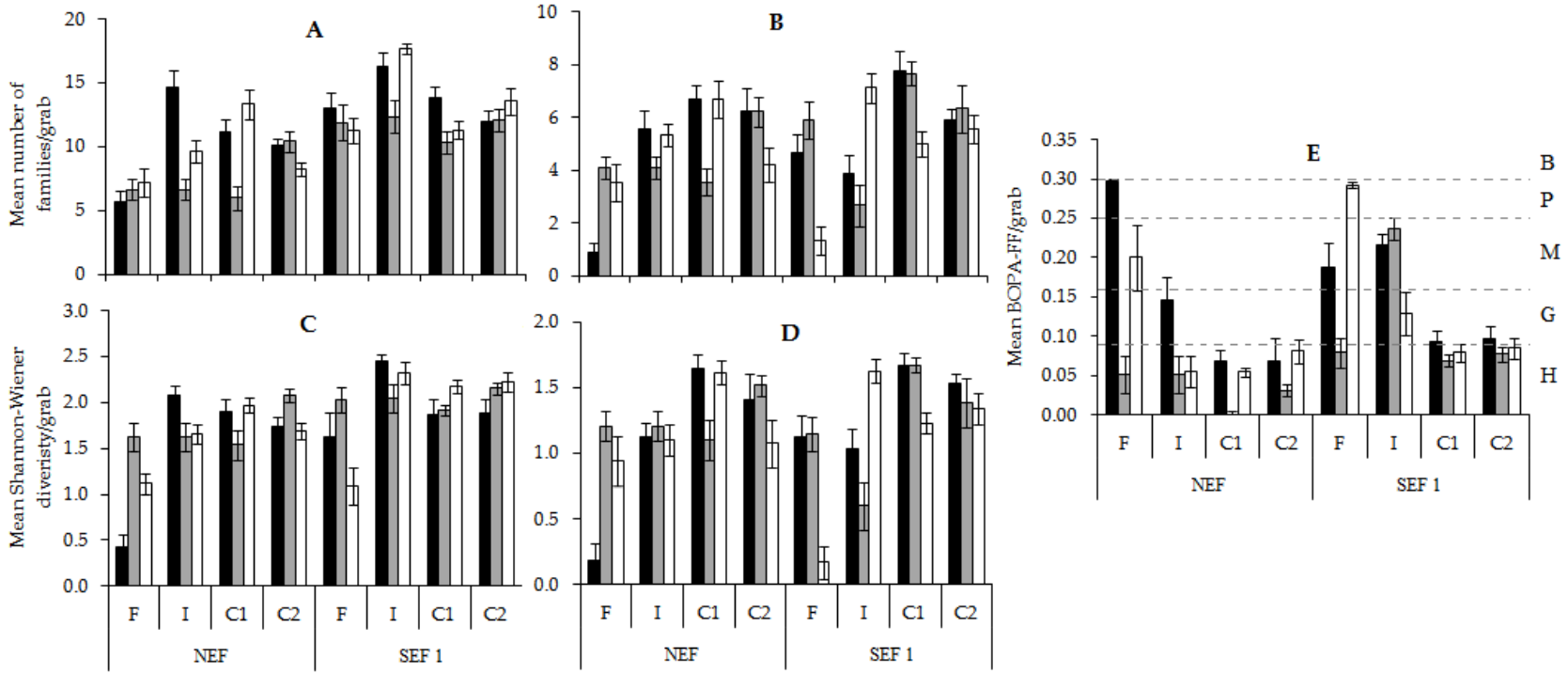


**Figure 5.4** Mean values (± SE) per grab of: (a, b) total number of families and (c, d) Shannon diversity of (a, c) polychaetes and (b, d) amphipods, and (e) polychaete/amphipod ratio recorded at the northeastern farm (NEF) and southeastern 'Farm 1' (SEF 1) farm (F), impacted (I), 'Control 1' (C1) and 'Control 2' (C2) plots in the years 2003 (black bars), 2004 (grey bars), and 2005 (white bars).

C1 C2', 'Ti x I-vs-C1 C2' and 'Ti x C1-vs-C2', and for 'Lo x F-vs-I C1 C2 x Ti', 'Lo x C1-vs-C2 x Ti' and 'Lo x I-vs-C1 C2 x Ti' (Table 5.3). On the other hand, PERMANOVA indicated significant difference in BOPA-FF for 'Si(Lo x F-vs-I C1 C2 x Ti)' ($p < 0.0001$), 'Si(Lo x I-vs-C1 C2 x Ti)' ($p < 0.001$), and 'Si(Lo x C1-vs- C2 x Ti)' ($p < 0.01$) (Table 5.4).

*A posteriori* PERMANOVA indicated a significant difference for: (i) 'Ti x F-vs-I C1 C2' in the abundance of Maldanidae ($p < 0.001$) and Phoxocephalidae ($p < 0.01$) at the NEF, and Ampeliscidae at 'Farm 2' ($p < 0.05$); and (ii) for 'Ti x C1-vs-C2' in the abundance of Phoxocephalidae and Sabellidae at the NEF ($p < 0.05$) (Table 5.5). Pair-wise tests showed a significantly lower ($p < 0.01$) abundance of: (i) Maldanidae and Phoxocephalidae (for 2003), and Phoxocephalidae (for 2005), at the farm plot compared to the impacted and control plots at the NEF, and (ii) abundance of Ampeliscidae at the farm plot compared to the impacted and control plots at the SEF 1 in 2003 (Table 5.5). Pair-wise tests also showed a significantly higher abundance of Maldanidae at the impacted plot compared to the control plots in 2003 ($p < 0.05$), and of Phoxocephalidae ($p < 0.001$) and Sabellidae ($p < 0.01$) at the 'Control 1' plot compared to the 'Control 2' plot in 2005 at the NEF (Table 5.5).

*A posteriori* PERMANOVA also indicated a significant difference for: (i) 'Si(F-vs-I C1 C2 x Ti)' in the abundance of Phoxocephalidae at the NEF ($p < 0.01$); (ii) 'Si(I-vs-C1 C2 x Ti)' in the abundance of Phoxocephalidae at the NEF ($p < 0.01$) and Ampeliscidae at the SEF 1 ($p < 0.01$); and (iii) 'Si(C1-vs-C2 x Ti)' in the abundance of Ampeliscidae at the SEF 1 ($p < 0.001$) (Table 5.5).

**Table 5.5** Results of three-factor univariate *a posteriori* PERMANOVA for number of individuals of taxa contributing most to the dissimilarity in polychaete and amphipod assemblages at incremental distances from the tuna pens, with planned contrast tests for the factor 'Distance', and the F-ratio numerator/s and denominator/s. Df = degrees of freedom, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', Mal = Maldanidae, Phox = Phoxocephalidae, Sab = Sabellidae, Amp = Ampeliscidae, RES = Residual, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | | NEF | | | SEF 1 | | |
|---|---|---|---|---|---|---|---|
| Source of Variation | df | Mal | Phox | Sab | Amp | F-ratio Numerator | F-ratio Denominator |
| Distance = Di | 3 | ns | ns | ns | * | Di | Ti x Di |
| F-vs-I C1 C2 | 1 | ns | ns | ns | * | F-vs-I C1 C2 | Ti x F-vs-I C1 C2 |
| I-vs-C1 C2 | 1 | ns | * | ns | ns | I-vs-C1 C2 | Ti x I-vs-C1 C2 |
| C1-vs-C2 | 1 | ns | ns | ns | ns | C1-vs-C2 | Ti x C1-vs-C2 |
| Time = Ti | 2 | ns | ns | ns | ns | Ti | Ti x Di |
| Ti x Di | 6 | * | * | ns | ns | Ti x Di | Si(Di x Ti) |
| Ti x F-vs-I C1 C2 | 2 | *** | ** | ns | * | Ti x F-vs-I C1 C2 | Si(F-vs-I C1 C2 x Ti) |
| Ti x I-vs-C1 C2 | 2 | ns | ns | ns | ns | Ti x I-vs-C1 C2 | Si(I-vs-C1 C2 x Ti) |
| Ti x C1-vs-C2 | 2 | ns | * | * | ns | Ti x C1-vs-C2 | Si(C1-vs-C2 x Ti) |
| Site(Di x Ti) = Si(Di x Ti) | 24 | * | * | ns | *** | Si(Di x Ti) | RES: Si(Di x Ti) |
| Si(F-vs-I C1 C2 x Ti) | 6 | ns | * | ns | ns | Si(F-vs-I C1 C2 x Ti) | RES: Si(F-vs-I C1 C2 x Ti) |
| Si(I-vs-C1 C2 x Ti) | 6 | ns | ** | ns | ** | Si(I-vs-C1 C2 x Ti) | RES: Si(I-vs-C1 C2 x Ti) |
| Si(C1-vs-C2 x Ti) | 12 | ns | ns | ns | *** | Si(C1-vs-C2 x Ti) | RES: Si(C1-vs-C2 x Ti) |
| RES: Si(Di x Ti) | 72 | | | | | | |
| RES: Si(F-vs-I C1 C2 x Ti) | 18 | | | | | | |
| RES: Si(I-vs-C1 C2 x Ti) | 18 | | | | | | |
| RES: Si(C1-vs-C2 x Ti) | 36 | | | | | | |
| Total | 107 | | | | | | |

**Table 5.5** Continued

| *Pair-wise tests -: 'Ti x Di'* | | | | | |
|---|---|---|---|---|---|
| Level | Groups | Mal | Phox | Sab | Amp |
| 2003 | F-vs-I C1 C2 | < ** | < ** | - | < ** |
| | I-vs-C1 C2 | > * | ns | - | ns |
| | C1-vs-C2 | ns | ns | - | ns |
| 2004 | F-vs-I C1 C2 | - | - | - | - |
| | I-vs-C1 C2 | - | - | - | - |
| | C1-vs-C2 | - | - | - | - |
| 2005 | F-vs-I C1 C2 | - | < ** | ns | - |
| | I-vs-C1 C2 | - | ns | ns | - |
| | C1-vs-C2 | - | > *** | > ** | - |

### 5.3.2 Multivariate data analyses

#### 5.3.2(i) Sediment physico-chemical attributes

The results of PCO ordination explained 57.1% of the total variation in sediment physico-chemical data and indicated similarity in sediment samples collected from the NEF and SEF 1 farm and control plots in 2003, 2004 and 2005, while sediment samples collected from the NEF impacted plot in 2003 and 2004, SEF 1 impacted plot in 2003, and SEF 1 impacted plot in 2004 and 2005, were grouped differently from one another (Figure 5.5).

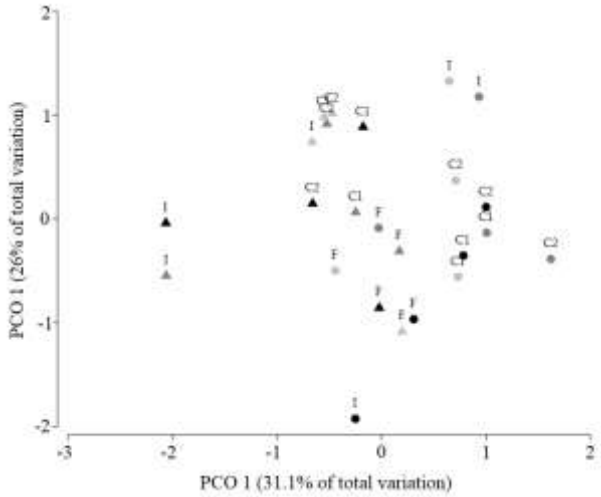


**Figure 5.5** PCO plot calculated from a Euclidean d of sediment physico-chemical attributes derived from normalised environmental data collected from the northeastern farm (circle) and southeastern 'Farm 1' (triangle) at farm (F), impacted (I), 'Control 1' (C1) and 'Control 2' (C2) plots in the years 2003 (black), 2004 (dark grey), and 2005 (light grey).

Multivariate PERMANOVA indicated that the square root estimates for the interaction terms 'Ti x F-vs-I C1 C2', 'Ti x I-vs-C1 C2' and 'Ti x C1-vs-C2', and 'Lo x F-vs-I C1 C2 x Ti', 'Lo x I-vs-C1 C2 x Ti' and 'Lo x C1-vs-C2 x Ti', as components of variation in sediment physico-chemical attributes, were small or negative (-0.09119 to 0.78618), while both PERMANOVA and PERMDISP tests indicated no significant difference in sediment physico-chemical attributes for 'Ti x F-vs-I C1 C2', 'Ti x I-vs-C1 C2' and 'Ti x C1-vs-C2', and for 'Lo x F-vs-I C1 C2 x Ti', 'Lo x I-vs-C1 C2 x Ti' and 'Lo x C1-vs-C2 x Ti' (Table 5.6).

**Table 5.6** Results of three-factor multivariate PERMANOVA and PERMDISP tests for sediment phyisco-chemical composition, with planned contrast tests for the factor 'Distance'. Variables in the analyses included normalised values of MSGS, POCC and PONC. The level of significance was set at 0.05. Df = degrees of freedom, Sq Rt Var = square root estimate of component of variation, RES = Residual, ns = not significant, * = $p < 0.05$

| | | PERMANOVA | | PERMDISP |
|---|---|---|---|---|
| Source of Variation | df | Sq Rt Var | p-value | p-value |
| Location = Lo | 1 | 0.7807 | ns | ns |
| Distance = Di | 3 | 0.4675 | ns | ns |
| F-vs-I C1 C2 | 1 | 0.1875 | ns | ns |
| I-vs-C1 C2 | 1 | 0.2122 | ns | * |
| C1-vs-C2 | 1 | 0.06769 | ns | ns |
| Time = Ti | 2 | 0.2633 | ns | ns |
| Lo x Di | 3 | 0.7736 | ns | ns |
| Lo x F-vs-I C1 C2 | 1 | 0.462 | ns | ns |
| Lo x I-vs-C1 C2 | 1 | 0.4109 | ns | * |
| Lo x C1-vs-C2 | 1 | -0.09937 | ns | * |
| Lo x Ti | 2 | 0.1297 | ns | ns |
| Ti x Di | 6 | 0.418 | ns | ns |
| Ti x F-vs-I C1 C2 | 2 | -0.09119 | ns | ns |
| Ti x I-vs-C1 C2 | 2 | 0.7862 | ns | ns |
| Ti x C1-vs-C2 | 2 | -0.277 | ns | ns |
| Lo x Di x Ti | 6 | 0.5711 | ns | ns |
| Lo x F-vs-I C1 C2 x Ti | 2 | -0.2228 | ns | ns |
| Lo x I-vs-C1 C2 x Ti | 2 | 0.3626 | ns | ns |
| Lo x C1-vs-C2 x Ti | 2 | 0.4313 | ns | ns |
| RES(Lo x Di x Ti) | 48 | 1.409 | | |
| RES(Lo x F-vs-I C1 C2 x Ti) | 12 | 0.1689 | | |
| RES(Lo x I-vs-C1 C2 x Ti) | 12 | 0.0232 | | |
| RES(Lo x C1-vs-C2 x Ti) | 24 | 1.217 | | |
| Total | 71 | | | |

### 5.3.2(ii) Macroinvertebrate assemblages

PCO ordination explained 67.5% of the total variation observed in polychaete family abundance, and showed separation of samples over time between the two tuna farms (Figure 5.6). Higher dispersion of samples characterised polychaete family abundances at the NEF compared to the SEF 1; samples collected in 2004 from the NEF appeared as a seperate group from samples collected in 2003 and 2005 from the same farm. PCO ordination explained 55.2% of the total variation observed in amphipod family abundance data, and showed high dispersion of samples collected

from farm and impacted plots at both tuna farms (Figure 5.6).Multivariate PERMANOVA indicated that the square root estimates for 'Ti x F-vs-I C1 C2', 'Ti x I-vs-C1 C2' and 'Ti x C1-vs-C2', and for 'Lo x F-vs-I C1 C2 x Ti', 'Lo x I-vs-C1 C2 x Ti' and 'Lo x C1-vs-C2 x Ti', as components of variation in the family- abundances of polychaetes (0.5955 to 4.245, and 0.1970 to 10.00, respectively) and amphipods (-4.007 to 7.903, and -1.371 to 15.39, respectively), were negative or small, compared to the square root estimate for the residual variation 'RES:Si(Lo x Pl x Ti)' (26.31 and 38.23, respectively) (Table 5.7). PERMDISP indicated significant differences ($p < 0.001$) for 'Ti x F-vs-I C1 C2', 'Ti x I-vs-C1 C2' and 'Ti x C1-vs-C2', and for 'Lo x F-vs-I C1 C2 x Ti', 'Lo x I-vs-C1 C2 x Ti' and 'Lo x C1-vs-C2 x Ti' in polychaete family abundances, and for 'F-vs-I C1 C2 x Ti', and 'I-vs-C1 C2 x Ti', and 'Lo x F-vs-I C1 C2 x Ti' and 'Lo x I-vs-C1 C2 x Ti', in amphipod family abundances; while PERMANOVA did not (Table 5.7).

Pair-wise tests showed that at the NEF dispersion of samples based on amphipod family abundance was significantly higher at the farm plot compared to the impacted and control plots in 2003 ($p < 0.0001$) and 2005 ($p < 0.01$), while dispersion of samples based on polychaete family abundance at the 'Control 1'plot was significantly higher ($p < 0.001$) compared to the 'Control 2' plot in 2004 (Table 5.7). At the SEF 1, dispersion of samples based on amphipod family abundance was significantly higher at the farm plot compared to the impacted and control plots in 2005 ($p < 0.001$); and at the impacted plot compared to the control plots in 2003 ($p < 0.05$), 2004 ($p < 0.01$) and 2005 ($p < 0.001$). Pair-wise tests also showed that dispersion of samples based on polycheate and amphipod family abundance at the farm plot was significantly higher ($p < 0.01$) at the NEF compared to the SEF 1, in 2003 and 2005, respectively (Table 5.7).

PERMDISP also showed significant differences for 'Si(Lo x F-vs-I C1 C2 x Ti)' ($p < 0.001$), 'Si(Lo x I-vs-C1 C2 x Ti)' ($p_{Polychaetes} < 0.001$, $p_{Amphipods} < 0.01$), and 'Si(Lo x C1-vs-C2 x Ti)' ($p_{Polychaetes} < 0.01$, $p_{Amphipods} < 0.05$) in the family abundance of polychaetes and amphipods (Table 5.7).

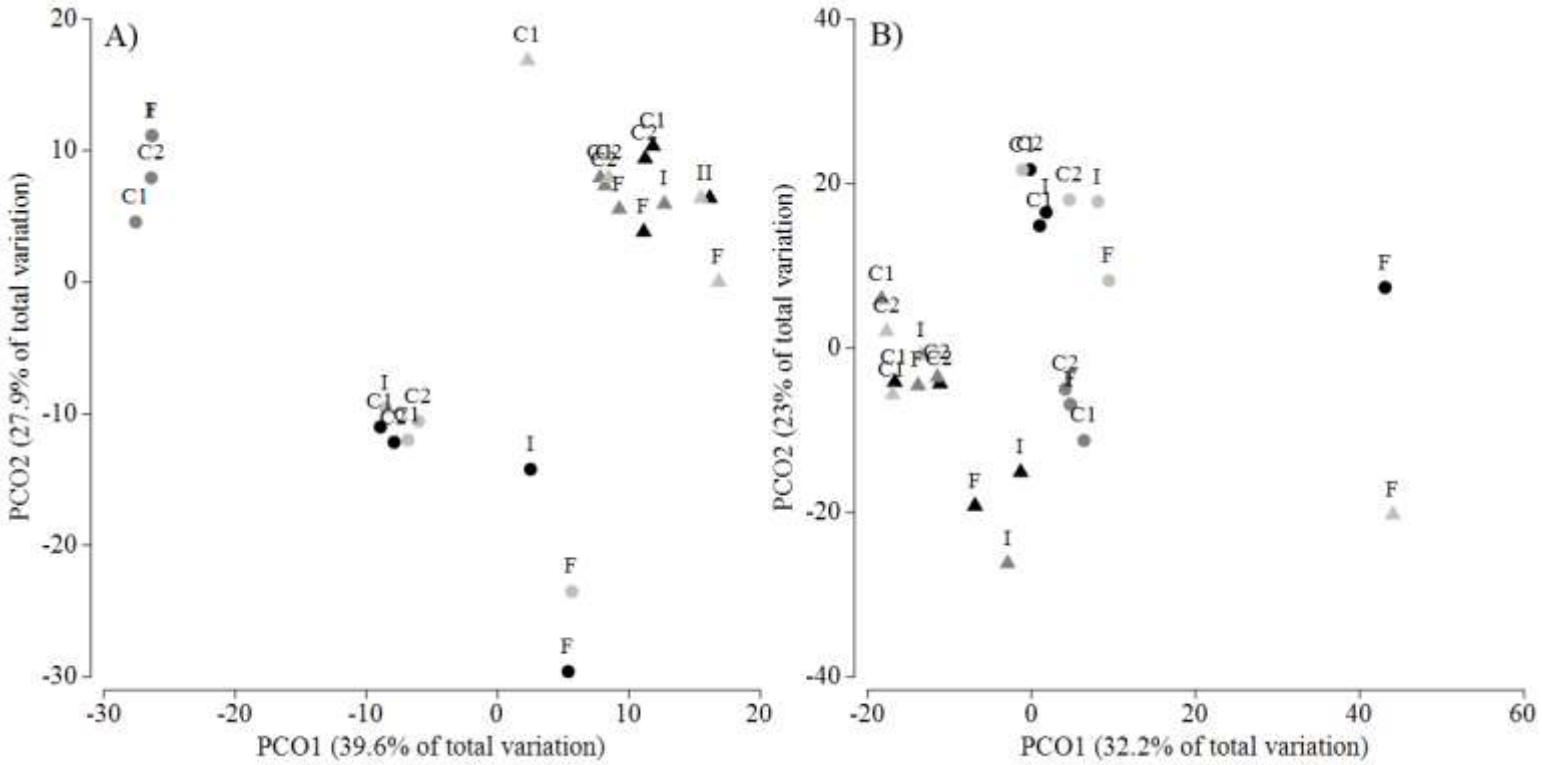


**Figure 5.6** Plots from PCO of fourth-root and square-root transformed family abundance values, respectively, of the polychaete (a) and amphipod (b) assemblages collected from the northeastern farm (circle) and southeastern 'Farm 1' (triangle) at farm (F), impacted (I), 'Control 1' (C1) and 'Control 2' (C2) plots in the years 2003 (black), 2004 (dark grey), and 2005 (light grey).

**Table 5.7** Results of four-factor multivariate PERMANOVA and PERMDISP tests for polychaete and amphipod assemblagess, with planned contrast tests for the factor 'Distance'. Variables included in the analysis are fourth-root and square-root transformed family abundance values of respectively polychaetes and amphipods. Df = degrees of freedom, Sq Rt Var = square root estimate of component of variation, RES = residual, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | | Polychaetes | | | Amphipods | | |
|---|---|---|---|---|---|---|---|
| | | PERMANOVA | | PERMDISP | PERMANOVA | | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value | Sq Rt Var | p-value | p-value |
| Location = Lo | 1 | 20.54 | ns | *** | 10.43 | ns | ns |
| Distance = Di | 3 | 11.30 | ns | * | 12.81 | ns | *** |
| F-vs-I C1 C2 | 1 | 4.342 | ns | * | 2.644 | ns | *** |
| I-vs-C1 C2 | 1 | 7.250 | ns | ns | 4.806 | ns | *** |
| C1-vs-C2 | 1 | -0.2960 | ns | ns | 5.364 | ns | ns |
| Time = Ti | 2 | 10.67 | ns | ** | 8.533 | ns | ns |
| Lo x Di | 3 | 7.735 | ns | *** | -5.364 | ns | *** |
| Lo x F-vs-I C1 C2 | 1 | 0.9047 | ns | *** | 0.1930 | ns | *** |
| Lo x I-vs-C1 C2 | 1 | 7.234 | ns | *** | 3.300 | ns | *** |
| Lo x C1-vs-C2 | 1 | -0.4040 | ns | *** | -8.857 | ns | ns |
| Lo x Ti | 2 | 16.47 | ns | ** | 10.40 | ns | ** |
| Ti x Di | 6 | 8.235 | ns | *** | 9.4220 | ns | *** |
| Ti x F-vs-I C1 C2 | 2 | 3.394 | ns | *** | 7.9034 | ns | *** |
| Ti x I-vs-C1 C2 | 2 | 4.245 | ns | *** | 5.526 | ns | *** |
| Ti x C1-vs-C2 | 2 | 0.5955 | ns | *** | -4.0071 | ns | ns |
| Lo x Di x Ti | 6 | 11.29 | ns | *** | 13.43 | ns | *** |
| Lo x F-vs-I C1 C2 x Ti | 2 | 0.1970 | ns | *** | -0.5980 | ns | *** |
| Lo x I-vs-C1 C2 x Ti | 2 | 10.00 | ns | *** | -1.371 | ns | *** |
| Lo x C1-vs-C2 x Ti | 2 | 1.0931 | ns | *** | 15.39 | ns | ns |

**Table 5.7** Continued

| | | Polychaetes | | | Amphipods | | |
|---|---|---|---|---|---|---|---|
| | | PERMANOVA | | PERMDISP | PERMANOVA | | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value | Sq Rt Var | p-value | p-value |
| Si(Lo x Di x Ti) = Si(Lo x Di x Ti) | 48 | 13.12 | ** | *** | 20.48 | ** | *** |
| Si(Lo x F-vs-I C1 C2 x Ti) | 12 | 1.067 | ns | *** | 1.987 | ns | *** |
| Si(Lo x I-vs-C1 C2 x Ti) | 12 | 11.3 | ns | *** | 5.46 | * | ** |
| Si(Lo x C1-vs-C2 x Ti) | 24 | 0.7432 | ns | ** | 13.03 | ns | * |
| RES: Si(Lo x Di x Ti) | 144 | 26.31 | | | 38.23 | | |
| RES: Si(Lo x F-vs-I C1 C2 x Ti) | 36 | 0.86 | | | 5.026 | | |
| RES: Si(Lo x I-vs-C1 C2 x Ti) | 36 | 23.32 | | | 3.122 | | |
| RES: Si(Lo x C1-vs-C2 x Ti) | 72 | 2.129 | | | 30.08 | | |
| Total | 215 | 125.7 | | | 118.4 | | |
| *Pair-wise tests -: 'Lo x Di x Ti'* | | | | | | | |
| | | | p(MC) | p(MC) | | p(MC) | p(MC) |
| NEF F-vs-I C1 C2 | 2003 | | - | ns | | - | > *** |
| | 2004 | | - | ns | | - | ns |
| | 2005 | | - | ns | | - | > ** |
| NEF I-vs-C1 C2 | 2003 | | - | ns | | - | ns |
| | 2004 | | - | ns | | - | ns |
| | 2005 | | - | ns | | - | ns |
| NEF C1-vs-C2 | 2003 | | - | ns | | - | - |
| | 2004 | | - | > *** | | - | - |
| | 2005 | | - | < * | | - | - |

**Table 5.7** Continued

| | | | Polychaetes | | Amphipods | |
|---|---|---|---|---|---|---|
| | | | PERMANOVA | PERMDISP | PERMANOVA | PERMDISP |
| Source of Variation | | df | Sq Rt Var p-value | p-value | Sq Rt Var p-value | p-value |
| *Pair-wise tests -: 'Lo x Di x Ti'* | | | | | | |
| SEF 1 | F-vs-I C1 C2 | 2003 | - | ns | - | ns |
| | | 2004 | - | ns | - | ns |
| | | 2005 | - | ns | - | > *** |
| | I-vs-C1 C2 | 2003 | - | ns | - | > * |
| | | 2004 | - | ns | - | > *** |
| | | 2005 | - | ns | - | > *** |
| | C1-vs-C2 | 2003 | - | < * | - | - |
| | | 2004 | - | ns | - | - |
| | | 2005 | - | ns | - | - |
| NEF vs SEF 1 | Farm | 2003 | - | ns | - | > ** |
| | | 2004 | - | ns | - | ns |
| | | 2005 | - | > * | - | ns |
| | Impact | 2003 | - | ns | - | ns |
| | | 2004 | - | ns | - | < ** |
| | | 2005 | - | ns | - | ns |
| | 'Control 1' | 2003 | - | ns | - | - |
| | | 2004 | - | > ** | - | - |
| | | 2005 | - | ns | - | - |
| | 'Control 2' | 2003 | - | ns | - | - |
| | | 2004 | - | ns | - | - |
| | | 2005 | - | > *** | - | - |

### 5.3.3 Relationship between sediment and macroinvertebrates

BEST analysis for the NEF showed significant correlation between: (i) POCC and abundance of Syllidae ($p < 0.05$, $\rho = 0.685$); and (ii) a combination of MSGS and POCC and Shannon-Wiener diversity of polychaetes at the farm plot (Table 5.8). At the impacted plot, there was significant correlation between: (i) a combination of POCC and PONC and abundance of Capitellidae ($p < 0.05$, $\rho = 0.637$); (ii) a combination of MSGS and PONC and abundance of Syllidae ($p < 0.05$, $\rho = 0.667$) and Dorvilleidae ($p < 0.05$, $\rho = 0.664$); and (iii) PONC and number of polychaete families ($p < 0.05$, $\rho = 0.664$) and polychaete/amphipod index ($p < 0.05$, $\rho = 0.605$). At the 'Control 2' plot, there was significant correlation between the abundance of Photidae and MSGS ($p < 0.05$, $\rho = 0.731$) (Table 5.8).

BEST analysis for the SEF 1 showed significant correlation between: (i) MSGS and the polychaete/amphipod index at the impacted plot ($p < 0.05$, $\rho = 0.673$); and (ii) MSGS and the number of polychaete families ($p < 0.05$, $\rho = 0.528$), and between PONC and the assemblage composition of polychaetes ($p < 0.05$, $\rho = 0.639$) at the 'Control 2' plot (Table 5.8).

BEST analysis indicated no significant correlation between sediment physico-chemical variables and attributes of the macroinvertebrate assemblages at the NEF control plots, and at the SEF 1 farm and 'Control 1' plots (Table 5.8).

**Table 5.8** Results of BEST analysis showing the environmental variable or combination thereof that best explains the variation in attributes of the macroinvertebrate assemblages recorded at incremental distances from the tuna pens per farm. A Euclidean similarity matrix was used for univariate biotic data, while a Bray Curtis similarity matrix of fourth-root transformed family abundance data was used for multivariate analyses. The level of significance was set at 0.05. Significant p-values shown in bold. NEF = northeastern farm, SEF 1 = southeastern 'Farm 1' Dep Var = dependent variable, ρ-value = Spearman's rank correlation coefficient, Best Exp Var = best explanatory variable, NI = number of individuals, NFa = number of families, ShW = Shannon-Wiener diversity; Polychaete indicator taxon 1 = Capitellidae, 2 = Syllidae, and 3 = Dorvilleidae; Amphipod indicator taxon 1 = Urothoidae, 2 = Photidae, and 3 = Lysianassidae; BOPA-FF = polychaete/amphipod (BOPA-Fish farming) index, AsC = assemblage composition, MSGS = mean sediment grain size, POCC = percent organic carbon content, PONC = percent organic nitrogen content, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$

| | | NEF | | | | SEF 1 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Polychaetes | | Amphipods | | Polychaetes | | Amphipods | |
| | Dep Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| Farm | NI 1 | 0.442, ns | POCC | 0.092, ns | MSGS | 0.137, ns | POCC | 0.234, ns | MSGS |
| | NI 2 | 0.685, * | POCC | 0.280, ns | PONC | 0.459, ns | POCC | 0.313, ns | MSGS, POCC |
| | NI 3 | 0.459, ns | POCC | 0.006, ns | MSGS, PONC | 0.019, ns | POCC | 0.090, ns | PONC |
| | NFa | 0.134, ns | POCC | -0.017, ns | MSGS | 0.490, ns | POCC | 0.108, ns | MSGS |
| | ShW | 0.490, * | MSGS,MSGS, POCC | 0.150, ns | POCC | 0.345, ns | MSGS, POCC, PONC | 0.207, ns | POCC, PONC |
| | AsC | 0.365, ns | PONC | 0.274, ns | POCC | 0.450, ns | PONC | 0.155, ns | PONC |
| Impact | NI 1 | 0.637, * | POCC, PONC | -0.030, ns | MSGS, POCC | 0.154, ns | POCC | 0.354, ns | MSGS |
| | NI 2 | 0.667, * | MSGS, PONC | 0.495, ns | MSGS, POCC | 0.090, ns | PONC | 0.187, ns | MSGS, POCC |
| | NI 3 | 0.690, * | MSGS, PONC | 0.594, ns | MSGS | 0.547, ns | POCC | 0.466, ns | MSGS, POCC |
| | NFa | 0.664, * | PONC | 0.419, ns | MSGS | 0.020, ns | POCC, PONC | 0.413, ns | MSGS, POCC |
| | ShW | 0.447, ns | PONC | 0.117, ns | PONC | 0.079, ns | POCC | 0.246, ns | MSGS |
| | AsC | 0.145, ns | MSGS, POCC | 0.466, ns | MSGS, POCC | -0.098, ns | PONC | -0.008, ns | POCC |
| 'Control 1' | NI 1 | 0.094, ns | MSGS | 0.549, ns | MSGS | 0.148, ns | MSGS | 0.054, ns | PONC |
| | NI 2 | 0.599, ns | MSGS, PONC | 0.035, ns | MSGS | 0.474, ns | POCC, PONC | 0.259, ns | MSGS, POCC |
| | NI 3 | 0.101, ns | MSGS | 0.347, ns | MSGS, PONC | 0.209, ns | MSGS, POCC | 0.125, ns | MSGS |
| | NFa | 0.295, ns | MSGS | 0.003, ns | POCC, PONC | 0.448, ns | PONC | 0.326, ns | POCC |

**Table 5.8** Continued

| | | NEF | | | | SEF 1 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Polychaetes | | Amphipods | | Polychaetes | | Amphipods | |
| | Dep Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| 'Control 2' | ShW | 0.025, ns | POCC | 0.057, ns | POCC | 0.388, ns | POCC | 0.304, ns | POCC |
| | AsC | -0.006, ns | MSGS | 0.195, ns | POCC, PONC | 0.186, ns | POCC | 0.338, ns | POCC, PONC |
| | NI 1 | 0.456, ns | POCC | 0.109, ns | PONC | 0.492, ns | MSGS, PONC | -0.032, ns | MSGS |
| | NI 2 | 0.028, ns | MSGS | 0.272, ns | MSGS | 0.383, ns | MSGS, POCC, PONC | 0.297, ns | MSGS, POCC |
| | NI 3 | 0.110, ns | PONC | 0.731, ** | MSGS | 0.398, ns | PONC | 0.280, ns | MSGS, POCC |
| | NFa | 0.288, ns | POCC | 0.195, ns | PONC | 0.528, * | MSGS | 0.135, ns | MSGS, POCC |
| | ShW | 0.097, ns | POCC | 0.305, ns | MSGS, PONC | 0.248, ns | MSGS, PONC | 0.362, ns | PONC |
| | AsC | 0.369, ns | MSGS, POCC, PONC | 0.439, ns | MSGS, PONC | 0.639, ** | PONC | 0.361, ns | MSGS, PONC |

| | | NEF | | SEF 1 | |
|---|---|---|---|---|---|
| | Level | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| P/A | Farm | 0.162, ns | MSGS | 0.045, ns | PONC |
| | Impact | 0.606, * | PONC | 0.673, * | MSGS |
| | Control 1 | -0.104, ns | POCC | 0.210, ns | PONC |
| | Control 2 | 0.384, ns | PONC | 0.044, ns | MSGS, POCC, PONC |

## 5.4 Discussion

Assessment of data from two different ABT farms collected over three years of farming operations showed a spatial pattern of stressed macroinvertebrate assemblages with a significant influence on the macrobenthic invertebrate assemblages associated with the seabed directly below the tuna cages at both investigated farms; such significant influence extends up to 100 m from the SEF 1 cages. Similar results are reported at other Mediterranean tuna farms with an impact radius of about 100-200 m (Vita & Marin, 2007). The rate of deposition of organic matter from the fish cages decreases with increasing distance from the farm (Holmer *et al*., 2007), such that the amount of organic matter accumulated in the sediments decreases with distance from fish cages (e.g. Karakassis, Tsapakis, & Hatziyanni, 1998; Papageorgiou *et al*., 2010; Pereira *et al*., 2004), limiting the spatial extent of impact to the vicinity of the fish farm (e.g. Di Marco *et al*., 2017; Srithongouthai & Tada, 2017; Tomassetti *et al*., 2016).

Studies at other Mediterranean tuna farms report large amounts of uneaten feed-fish and tuna faeces (Aguado *et al*., 2004; Aguado-Giménez *et al*., 2006; Borg & Schembri, 2005; Mangion *et al*., 2014; Vita & Marin, 2007; Vita *et al*., 2004), organic carbon (Mangion *et al*., 2014; Marin *et al*., 2007; Matijević *et al*., 2006; Vita & Marin, 2007), and nitrogen (Mangion *et al*., 2014; Matijevic *et al*., 2006) in the sediment below fish cages, and a low silt-clay fraction that peaks at an intermediate distance from a fish farm (Marin *et al*., 2007; Vita & Marin, 2007). Present results show significantly high PONC at a distance of 100 m from the cages at both tuna farms, while MSGS and POCC did not differ significantly with increasing distance from the farms during the study period.

Capitellidae are opportunistic polychaetes (Borja *et al*., 2000) notoriously abundant below fish farms (e.g. Brown, 1987; Dean, 2008; Karakassis *et al*., 2000; Mangion *et al*., 2014; Vita & Marin, 2007) and act as good indicators of organic pollution. Dorvilleidae polychaetes are tolerant to fish farm pollution (Martinez-Garcia *et al*., 2013), while other polychaete families including Maldanidae (Martinez-Garcia *et al*., 2013) and Syllidae (Giangrande *et al*., 2005), as well as malacostracan crustaceans, including amphipods (Sanz-Lazáro & Marin, 2011), are sensitive to organic

enrichment. Some workers have applied the polychaete/amphipod ratio to assess the EQS of coastal waters under the influence of fish farming activities (e.g. Aguado-Giménez *et al.*, 2015; Jahani *et al.*, 2012; Mangion *et al*., 2017; Moraitis *et al*., 2013).

Studies have reported low species diversity and richness below fish cages, with a peak in diversity and number of species at intermediate distances from farms rearing Atlantic salmon (e.g. Brown *et al*., 1987; Edgar, Macleod, Mawbey, & Shields, 2005; Kutti *et al*., 2007a; Nickell *et al*., 2003), while a similar pattern has been reported for farms that grow sea bream and sea bass in the Mediterranean (Karakassis *et al*., 2000). Other works that assessed the EQS in the immediate vicinity of fish farms reported 'Bad' EQS in the Arabian Sea (Jahani *et al*., 2012) and 'Moderate' EQS in the Mediterranean Sea (Aguado-Giménez *et al*., 2015). Brown *et al*. (1987) reported significant correlation between the distribution of species along a spatial gradient of organic enrichment, and of sediment particle size along the same gradient, at a salmon farm in a Scottish sea loch. In the Mediterranean, workers have described polychaete abundance, richness and diversity that was influenced by a combination of sediment nitrogen content, sulfide content and silt-clay fraction (Martinez-Garcia *et al*., 2013), as well as a crustacean abundance that was influenced by sediment sulfide levels (Sutherland *et al*., 2007) at fish farm sites. De-la-Ossa-Carretero, Del-Pilar-Ruso, Gimenez-Casalduero, and Sanchez-Lizaso (2016) noted that distribution of amphipods in areas influenced by multiple anthropogenic stressors depended on sediment grain size and organic matter content.

With respect to Mediterranean tuna farms, Vita and Marin (2007) described a spatial gradient in macrofaunal assemblages characterized by a high impact area in the immediate vicinity of the cages, a high density of opportunistic species up to a distance of 40 m from the cages, and moderately stressed assemblages up to a distance of 200 m from the cages. At the same tuna farms studied in the present work, Mangion *et al*. (2017) reported EQS values that varied from 'Bad'/'Moderate' to 'Good', and significant correlation between sediment POCC, and Capitellidae abundance and number and Shannon-Wiener diversity of amphipod taxa in the immediate vicinity of the cages, from analysis of data collected over a three year study period. On the other hand, Moraitis *et al*. (2013) recorded 'Good' and 'High' EQS at other Mediterranean tuna farms, which was attributed to the high exposure of the farm sites.

Present findings at both investigated tuna farms showed a significantly higher abundance of Capitellidae and a significantly lower abundance and number of amphipod families, and Shannon-Wiener diversity of polychaetes and amphipods below the cages. 'Bad' and 'Poor' EQS was recorded at the NEF, and 'Bad' and 'Moderate' EQS was recorded at the SEF 1. Furthermore, dispersion of samples based on amphipod abundance that is indicative of stressed assemblages (e.g Stark *et al*., 2003; Warwick & Clarke, 1993) was significantly high below the cages of both tuna farms. Results also showed significantly lower abundance of Maldanidae below the cages of the NEF. At a distance of 100 m from the fish cages, the abundance of amphipods was significantly low at both tuna farms, while significantly lower Shannon-Weiner diversity of amphipods was recorded only at the NEF.

Differences in the pattern of sediment loading with distance from fish farms may result from differences in farm management practices and characteristics of the installations, as well as on the hydrodynamic regime of the area (Sanz-Lázaro & Marin, 2011). Fernandez-Gonzalez *et al*. (2013) previously reported high variation in sediment characteristics and attributes of amphipod assemblages at multiple spatial scales, at Mediterranean farms culturing sea bass and sea bream. The two tuna farms included in the present study differed in size, stocking density and feed management, as well as their location; hence one would expect differences in the pattern of influence on the seabed between them (Mangion *et al*., 2018). Mangion *et al*. (2018) previously reported differences in the magnitude and spatial extent of influence among the farms, which was attributed to differences in the total annual production of the farms and characteristics of the receiving environment (Borja *et al*., 2009c). In the present study, higher accumulation of fish-bones on the seabed below the cages of the SEF 1, and significantly higher levels of POCC and PONC 100 m away from the cages of the same farm, indicated that sediment loading was higher at this farm compared to the NEF. A significant peak in the Shannon-Wiener diversity of amphipods at intermediate distance from the cages (100 m) as described by the Pearson-Rosenberg model, was recorded during the study period at the SEF 1. No peak in diversity at intermediate levels of enrichment was recorded at the NEF; this is in concordance with results obtained by Vita and Marin (2007) at other Mediterranean tuna farms. On the other hand, significant correlation between MSGS, and sediment POCC and PONC, and attributes of the polychaete and amphipod assemblages, was recorded within a 100

m radius of the NEF, but not below the tuna cages of the SEF 1. Papageorgiou *et al*. (2010) previously noted no consistent pattern in macrofaunal diversity as a function of distance from fish cages between different study areas. Differences in sediment composition and functioning between different areas may not allow a maximal response of macroinvertebrate assemblages to fish farm wastes (Papageorgiou *et al*., 2010). It is also possible that the distance between sampling sites in the present study was too large to enable detection and characterisation of the spatial gradient in attributes of the macroinvertebrate benthic assemblages (Sanz-Lázaro & Marín, 2011).

Temporal variation in the deposition of organic matter on the seabed resulting from fish farming activities and the resultant benthic influence may arise from temporal variation in the fish farm feeding management strategy, as this determines the actual amount of feed consumed, and the amount of feed entering the marine environment, hence level of organic enrichment (Jansen *et al*., 2016; Kutti *et al*., 2007a). While previous studies examining the temporal pattern in macrofaunal diversity following cessation of fish farming activities have been carried out (e.g. Keeley, Forrest, & Macleod, 2015; Keeley, Macleod, Hopkins, & Forrest, 2014; Pereira *et al*., 2004; Salvo *et al*., 2017; Sanz-Lázaro & Marin, 2006; Zhulay, Reiss, & Reiss, 2015), it appears that no studies examining the temporal pattern in benthic assemblages during several fish farm production cycles have been published to date. Present results indicate temporal variation in the pattern of tuna penning influence on the seabed. Significantly lower number of amphipod families and diversity of polychaete and amphipod assemblages, and significantly higher dispersion based on polycheate and amphipod family abundance in the vicinity of the NEF indicate a higher level of influence there compared to the SEF 1. On the other hand, significantly higher abundance of Capitellidae and significantly lower number and Shannon-Weiner diversity of amphipod families recorded at times below the cages at the SEF 1 compared to the NEF, is indicative of the opposite pattern. Furthermore, the spatial pattern of stressed macroinvertebrate assemblages in the vicinity of the farms was not evident in 2004, when ‘Good’ EQS was recorded within the 100 m radius of the NEF, and in the immediate vicinity of the SEF 1; which may be attributed to improved feed management in that period.

The spatial extent of influence also differed between the two farms and appeared to be larger at the SEF 1 compared with the NEF; 'Poor' EQS and significant correlation between MSGS and the polychaete/amphipod index were recorded at the impacted plot located 100 m away from the cages at the SEF 1, while the EQS at the NEF impacted plot was 'Good' throughout the study period. The significant influence of tuna penning, indicated by significant correlation between MSGS and sediment PONC, and attributes of the polychaete assemblage, was also detected at the control area located 2 km up-current from the SEF 1; the acquired sea current data indicated a predominantly southern current (189 º) having a mean velocity of 0.185 ms $^{-1}$ in the vicinity of the southeastern farm site. These observations support the results obtained by Mangion *et al*. (2018) who noted an 'additive' effect of another tuna farm in the vicinity of the SEF 1 (1 km away), and highlighted the influence of higher nutrient loading of coastal waters in the southern parts of the Maltese Islands, as compared to their coastal waters in the north. Other studies similarly reported significant levels of organic waste in the sediment up to 1-2 km away from fish farm sites (e.g. Mangion *et al*., 2014; Puhr *et al*., 2017; Sarà, Scilipoti, Mazzola, & Modica, 2004; Wu *et al*., 1995).

# CHAPTER 6

# ASSESSING THE SUITABILITY OF BENTHIC BIOITC INDICES IN EVALUATION OF THE ECOLOGICAL INFLUENCE OF TUNA PENNING IN THE MALTESE ISLANDS

## 6.1 Introduction

European Union (EU) member states are obliged under the Water Framework Directive 2000/60/EC (WFD) to reach 'Good' Ecological Quality Status (EQS) for all water bodies, including coastal ecosystems, by 2015 (Directive, 2000). The need to assess the EQS has led to the evaluation of the performance of a variety of benthic biotic indices (BBIs) in the assessment of different types of environmental disturbances, in different habitats, and geographical regions (see Borja & Dauer [2008] for a review). A plethora of such indices are available at present, so the trend is to evaluate the suitability of these existing indices rather than develop new ones (e.g. Borja & Dauer, 2008; Borja *et al*., 2009b; Diaz, Solan, & Valente, 2004).

Indicator taxa indices are derived using data on the composition of macroinvertebrate assemblages along a gradient of increasing benthic disturbance, based on the concept of the sensitivity/tolerance of taxa making up an assemblage, to organic enrichment (e.g. Borja *et al*., 2000; Dauvin & Ruellet, 2007; Muxika *et al*., 2007; Simboura & Zentos, 2002). Tolerant taxa usually dominate low ecological quality habitats, while sensitive taxa are restricted to habitats of high ecological quality (Labrune *et al*., 2012). Indicator taxon indices developed under the WFD include the Azti-Marine Biotic Index (AMBI) (Borja *et al*., 2000), the BENTIX index (Simboura & Zenetos, 2002), and the Benthic Opportunistic Polychaetes and Amphipods index (BOPA) (Dauvin & Ruellet, 2007). These indices differ mainly in the weighting given to the relative abundance of taxa belonging to different ecological groups (EGs) of sensitivity/tolerance to disturbance (Labrune *et al*., 2012). However, it is difficult to to evaluate the density at which indicator taxa may be found in a community that has not been altered by human disturbance, since tolerant taxa may occur naturally in high densities, hence introducing an element of subjectivity in indicator taxa indices (Salas *et al*., 2006). Conversely, traditional diversity indices such as the Shannon-Wiener diversity index, are dependent on sample size, sampling effort, and habitat type; while indicator taxa indices are not (Subida *et al*., 2012). Indicator taxa indices give more reliable responses than diversity indices in assessment of moderate increases in organic matter levels in oligotrophic sediments such as those found in the Mediterranean Sea, since diversity shows a non-monotonic response to a gradient of organic enrichment, according to the Pearson and Rosenberg (1978) model in these conditions, particularly

at the low end of the spectrum (Subida *et al*., 2012). Multi-metric tools such as the Multivariate-AMBI (M-AMBI) index (Borja *et al*., 2004b; Muxika *et al*., 2007) combine the indicator taxa approach with diversity indices, and have been proposed to evaluate the EQS under the WFD (Borja, Muxika, & Rodriguez, 2009a).

The performance and agreement of various soft bottom, macroinvertebrate BBIs, developed for the implementation of the WFD, has been compared in different geographical regions for different types of environmental disturbances (e.g. Aguado-Giménez *et al*., 2007; Borja & Dauer, 2008; Borja, Marin, Rosa, & Muxika, 2014; Borja *et al*., 2009b, c; Çağlar & Albayrak, 2012; Dauvin *et al*., 2012; Dauvin, Ruellet, Desroy, & Janson, 2007; Fleischer *et al*., 2007; Grémare *et al*., 2009; Katsiaras *et al*., 2010; Keeley *et al*., 2012; Labrune *et al*., 2006, 2012; Marín-Guirao, Cesar, Marín, Lloret, & Vita, 2005; Simboura, 2004; Simboura & Argyrou, 2010; Simonini *et al*., 2009; Spagnolo *et al*., 2014; Subida *et al*., 2012; Wu *et al*., 2013). While different BBIs usually reflect the same general pattern of ecological quality and are significantly inter-correlated (e.g. Dauvin *et al*., 2007, 2012; Karakassis *et al*., 2013; Subida *et al*., 2012), there is often disagreement in the EQS assigned to individual sampling sites (e.g. Aguado-Giménez *et al*., 2007; Bouchet & Sauriau, 2008; Dauvin *et al*., 2007; Labrune *et al*., 2012; Simboura, 2004; Simboura & Argyrou, 2010; Wu *et al*., 2013). The selection of an appropriate index, classification of taxa according to their tolerance/sensitivity level, transferability to different biogeographical regions, link with causative environmental stressors, and definition of reference conditions, may prove problematic in assessment of environmental impacts using BBIs (e.g. Borja & Dauer, 2008; Martínez-Crego, Alcoverro, & Romero, 2010; Pinedo, Jordana, & Ballesteros, 2014; Simboura, 2004; Simboura & Argyrou, 2010; Teixeira *et al*., 2012). The use of taxa known locally to be indicators of fish farm pollution has been suggested to improve the performance of the BOPA index in aquaculture environmental impact monitoring (Aguado-Giménez *et al*., 2015). On the other hand, knowledge on the ecological strategies of local taxa may be lacking (e.g. Forde, Shin, Somerfield, & Kennedy, 2013; Keeley & Macleod, 2010). The taxonomic sufficiency principle has been applied recently to the Benthic Quality Index (Dimitriou *et al*., 2012) and BOPA index (Agaudo-Giménez *et al*., 2015) in an effort to decrease the taxonomic effort and cost of monitoring under the WFD, which allows for assessment of broader marine areas (Karakassis *et al*., 2013). The M-AMBI index at family level

has been suggested to apply the concept of tolerance/sensitivity levels at higher taxonomic levels when the existing knowledge on the ecological strategy of species is limited (Forde *et al*., 2013).

Aquaculture is usually carried out in waters with no or minimal sources of pollution, and typically constitutes several farming operations in one area, providing for robust comparison of different BBIs on a regional scale (Keeley *et al*., 2012). Data sets on the benthic influence of fish farming activities usually comprise information collected over a well-defined gradient of sediment loading over a relatively short distance (Karakassis *et al*., 2013). Previous studies have assessed the performance of BBIs developed for the implementation of the WFD to detect the influence of fish farming on the environment (e.g. Aguado-Giménez *et al*., 2007; Borja *et al*., 2009c; Edgar *et al*., 2010; Karakassis *et al*., 2013; Katsiaras *et al*., 2010; Muxika *et al*., 2005), as well as to assess the environmental effects of shell fish farming (e.g. Borja *et al*., 2009c; Bouchet & Sauriau, 2008; Callier, McKindsey, & Desrosiers, 2008). Workers have also compared the performance of these indices with traditional data analysis procedures used in aquaculture environmental impact monitoring studies (Aguado-Giménez *et al*., 2007). AMBI has been internationally proposed as a primary indicator of benthic health under the influence of fish farming (DSRSA, 2010), while multivariate analysis of physico-chemical and macrobenthic data is traditionally used for the environmental monitoring of fish farm activities (Aguado-Giménez *et al*., 2007). Good indicators of biological change potentially resulting from fish farming activities are the polychaete (e.g. Aguado-Giménez *et al*., 2015; Martinez-Garcia *et al*., 2013; Nobrega-Silva *et al*., 2016; Sutherland *et al*., 2007; Tomassetti & Porrello, 2005) and amphipod (e.g. Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011; Mangion *et al*., 2017) faunal groups.

Previous work carried out in Malta on local implementation of the WFD consisted of the application of AMBI and M-AMBI to coastal waters (MEPA, 2013). However, no studies comparing the local performance of BBIs developed for implementation of the WFD has been carried out to date, to the best of the present author's knowledge. The main aim of the present study was to assess the suitability of various BBIs; namely the AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices; for use in coastal waters of the Maltese Islands, by comparing their performance with analysis of data on

polychaete and amphipod assemblages that is traditionally used in aquaculture impact monitoring studies. Data on sediment quality and macroinvertebrate assemblages associated with the 'bare sand' habitat in the vicinity of two large tuna farms located circa 1 km off the coast of Malta, was used in the present assessment. The principle of taxonomic sufficiency is applied at the family level in the present data analyses.

## 6.2 Material and methods

### 6.2.1 Study sites and sampling

The two tuna farms considered in the present study were located 1 km off the northeastern to southeastern coast of the Maltese Islands (Figure 6.1), where the seabed consisted of soft sediment, and the water depth ranged from 42 m to 53 m. The northeastern farm (NEF) had eight tuna cages having a maximum total annual capacity of 2500 t, while the southernmost farm (southeastern 'Farm 1' [SEF 1]) was smaller and had three tuna cages having a maximum total annual capacity of 1500 t (ICCAT, 2011). Tuna penning operations started in summer in 2001 and 2002 respectively at the NEF and the SEF 1. The two farms utilized cages that had a diameter of some 50 m and a height of around 25 m. The farm lease areas were 350 m x 500 m (NEF) and 550 m x 550 m (SEF 1). The tuna were farmed at a stocking density of circa 2 to 4 kg $m^{-3}$, and were fed the equivalent of 3-4% of the fish biomass per day, in two feeding sessions (tuna farm managers, personal communication, January 14, 2015). The tuna were fed bait fish; namely squid, prawn, mackerel and sardines; and the food conversion ratio (based on the wet weight of the feed) was around 10-15:1 (tuna farm managers, personal communication, January 14, 2015).

The sampling design incorporated four sampling plots, which supported the same habitat type, at a similar water depth: (i) 'farm' plot; i.e. the seabed area occupied by the footprint of the tuna cages; (ii) 'impacted' plot; i.e. the seabed area c. 100 m around the tuna farm; (iii) 'Control 1' plot, located c. 1 km away from the cages; and (iv) 'Control 2' plot, located c. 2 km away from the cages (Figure 6.1). Three sampling sites were allotted to each plot, as the smallest farm had three cages. This sampling design was replicated at each of the two farms, such that it included a total of 24 sampling sites.

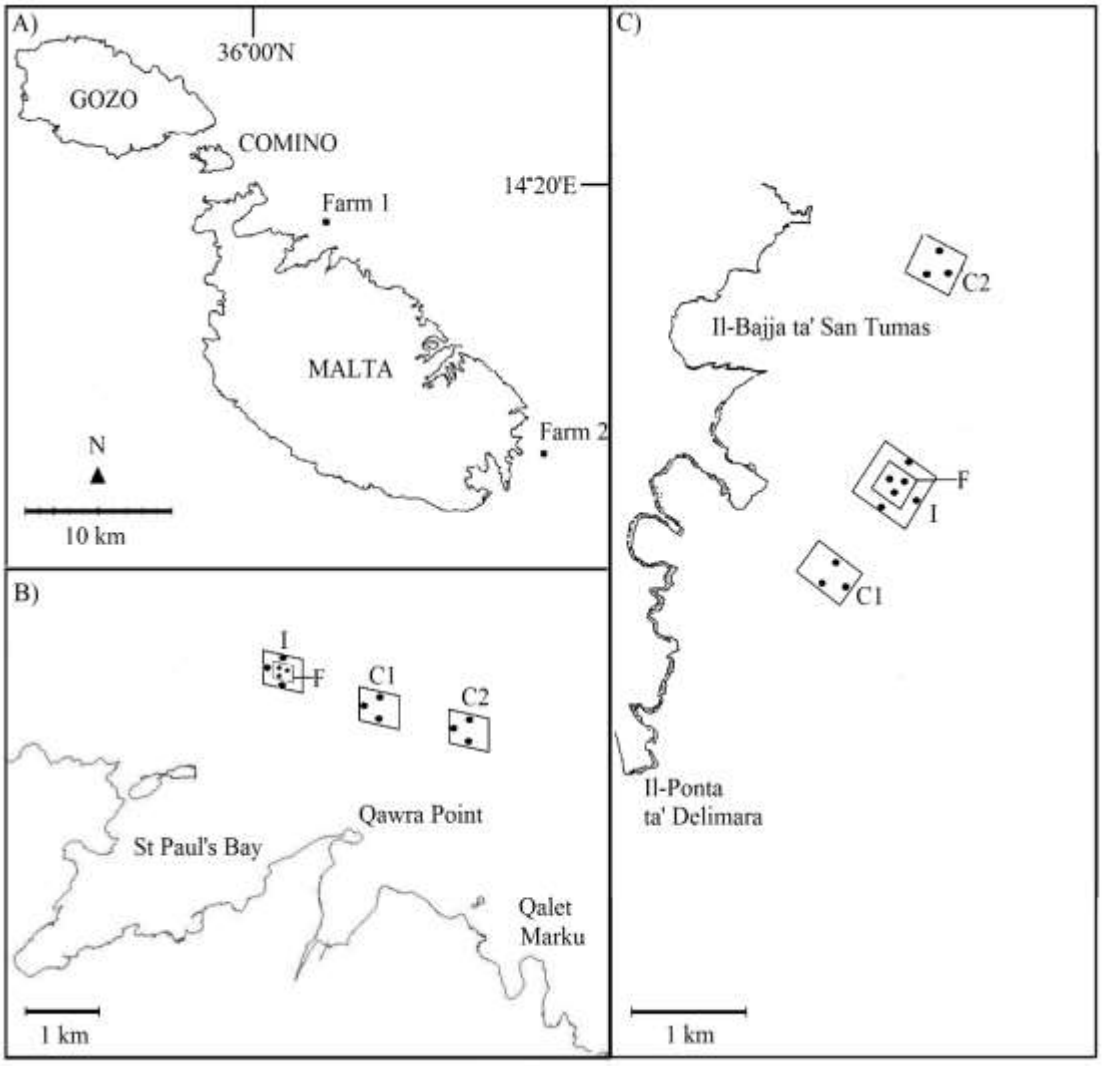


**Figure 6.1** Map of the Maltese Islands showing: (a) locations of the two tuna farms and the four sampling plots at the northeastern farm (b) and southeastern 'Farm 1' (c) from where the samples for benthic macrofaunal studies were collected. F = farm plot, I = impacted plot, C1 = 'Control 1' plot, C2 = 'Control 2' plot

Sampling of soft sediment assemblages was carried out in November 2003, 2004 and 2005 at the NEF, and in October 2003, 2004 and 2005 at the SEF 1, after initiation of tuna penning activities. Sampling was carried out using a 0.1 $m^2$ van Veen grab. Three replicate grab samples for benthic macrofaunal studies and one grab sample for sediment studies were collected at each of the 24 sampling sites. The latitude/longitude coordinates and water depth of the 24 sampling sites are given in Table 6.1. The collected samples were live-sieved using a 0.5 mm mesh, on board the vessel and temporarily preserved in 10 % formalin.

**Table 6.1** Latitude/longitude coordinates and depth of sites allotted to the impacted and control plots shown in Figure 6.1. The farm plot at the northeastern farm (NEF) was centered on: N35º 58.66'/E14º 25.16'; and at southeastern 'Farm 1' (SEF 1) at N35º 50.17'/E14º 35.11'; while samples were also collected from the seabed area directly below the cages.

| NEF | | | |
|---|---|---|---|
| Plot | Site | Latitude/Longitude | Depth (m) |
| Impact | S1 | N35º 58.77/E14 º 25.18 | 48 |
| | S2 | N35º 58.63/E14º 25.31 | 48 |
| | S3 | N35º 58.56/E14º 25.18 | 48 |
| 'Control 1' | S1 | N35º 58.51/E14º 26.33 | 52 |
| | S2 | N35º 58.38/E14º 26.47 | 52 |
| | S3 | N35º 58.32/E14º 26.33 | 51 |
| 'Control 2' | S1 | N35º 58.32/E14º 26.72 | 50 |
| | S2 | N35º 58.18/E14º 26.85 | 50 |
| | S3 | N35º 58.12/E14º 26.72 | 48 |
| SEF 1 | | | |
| Plot | Site | Latitude/Longitude | Depth (m) |
| Impact | S1 | N35º 50.61/E14º 35.16 | 50 |
| | S2 | N35º 50.52/E14º 35.25 | 51 |
| | S3 | N35º 50.52/E14º 35.00 | 46 |
| 'Control 1' | S1 | N35º 50.18/E14º 34.79 | 51 |
| | S2 | N35º 50.10/E14º 34.85 | 51 |
| | S3 | N35º 50.11/E14º 34.70 | 46 |
| 'Control 2' | S1 | N35º 51.58/E14º 35.42 | 47 |
| | S2 | N35º 51.50/E14º 35.48 | 47 |
| | S3 | N35º 51.49/E14º 35.37 | 45 |

In the laboratory, samples for faunal studies were sorted for polychaetes, molluscs, amphipods, decapods, and echinoderms after washing on a 0.5 mm mesh. Macroinvertebrates were identified to the family level (see Karakassis & Hatziyanni, 2000; Olsgard & Somerfield, 2000) and enumerated to obtain estimates of number of families and abundance per grab sample. For sediment physico-chemical studies, sub-samples for the determination of w/w feed-fish bone content (FFBC), percent organic carbon content (POCC), and percent organic nitrogen content (PONC) were frozen at

– 20 °C for later analysis. Another sub-sample was oven-dried for determination of mean sediment grain size (MSGS).

Analysis of the sediment to determine the FFBC was carried out by sorting fish bones from the sediment under a dissecting microscope. Sediment POCC was determined by wet oxidation using a chromic acid-sulfuric acid mixture, and titration of the evolved carbon dioxide (see Walkley & Black, 1934). PONC in the sediment was determined by the Kjeldhal method of digestion using concentrated sulfuric acid with a copper sulfate catalyst, followed by the addition of excess strong alkali, and condensation of the ammonia given off for titration. MSGS of the sediment was determined according to Buchanan (1984).

### 6.2.2 Data analyses

To calculate AMBI, BENTIX, BOPA, and M-AMBI indices at the family level, invertebrate families that are novel to the AMBI list (version June 2017) were assigned EGs calculated as the median EG of all taxa found in the AMBI list within the parent family, following Forde *et al*. (2013), and the EG classification was confirmed using Best Professional Judgment (see Teixeira *et al*., 2012) (Table 6.2).

AMBI was calculated using: AMBI = {(0 x %GI) + (1.5 x %GII) + (3 x %GIII) + (4.5 x %GIV) + (6 x %GV)}/100; where 'GI' are sensitive taxa, 'GII' are indifferent taxa, 'GIII' are tolerant taxa, 'GIV' are second-order opportunistic taxa, and 'GV' are first-order opportunistic taxa (Borja *et al*., 2000). BENTIX was calculated using: BENTIX = {6 %GI + 2(%GII + %GIII)}/100; where 'GI' are sensitive taxa, 'GII' are tolerant and second order opportunistic taxa, and 'GIII' are first order opportunistic taxa (Simboura & Zenetos, 2002). BOPA was calculated using: BOPA = log (($f_P$ / $f_A$ +1) +1); where '$f_P$' is the frequency of opportunistic polychaete individuals, and '$f_A$' is frequency of amphipod individuals excluding the genus *Jassa* (Dauvin & Ruellet, 2007). The BOPA-FF index was calculated using a modification of the BOPA index, in which the frequency of opportunistic polychaete individuals is replaced by the frequency of polychaetes tolerant to organic enrichment resulting from fish farm activities as identified by Martinez-Garcia *et al*. (2013) (Aguado-Giménez *et al*., 2015). The M-AMBI index was then calculated using reference values that represented

**Table 6.2** Invertebrate families that are novel to the AMBI list (version June 2017), with the proposed ecological group classification calculated as the median ecological group of all taxa within the parent family as listed in the AMBI list, and confirmed by Best Professional Judgement (see Forde *et al*., 2013). EG = Ecological Group, Cru = Crustacea, Gas = Gastropoda, Pol = Polychaeta, Sip = Sipuncula, Lep = Leptocardii, Ppc = Polyplacophora, Biv = Bivalvia, Ech = Echinodermata, Sca = Scaphopoda, I = sensitive taxa, II = indifferent taxa, III = tolerant taxa, IV = second-order opportunistic taxa, V = first-order opportunistic taxa

| Family | EG | Family | EG | Family | EG | Family | EG |
|---|---|---|---|---|---|---|---|
| Aplysiidae (Gas) | I | Epialtidae (Cru) | I | Majidae (Cru) | II | Pontoporeiidae (Cru) | I |
| Aporrhaidae (Gas) | I | Epitoniidae (Gas) | I | Mangeliidae (Gas) | I | Propeamussiidae (Biv) | I |
| Aspidosiphonidae (Sip) | II | Fasciolariidae | I | Marginellidae (Gas) | I | Pyramidellidae (Gas) | II |
| Asterinidae (Ech) | I | Fustiariidae (Sca) | I | Mitridae (Gas) | I | Raphitomidae (Gas) | I |
| Branchiostomatidae (Lep) | I | Gadilidae (Sca) | II | Muricidae (Gas) | I | Ringiculidae (Gas) | I |
| Caecidae (Gas) | I | Galatheidae (Cru) | I | Nassariidae (Gas) | II | Scalibregmatidae (Pol) | II |
| Callochitonidae (Ppc) | II | Glycymerididae (Biv) | III | Neolampadidae (Ech) | I | Scaphandridae (Gas) | II |
| Carditidae (Biv) | I | Gnathiidae (Iso) | I | Ophiocomidae (Ech) | I | Schizasteridae (Ech) | II |
| Cerithiidae (Gas) | II | Gryphaeidae (Biv) | II | Ophiomyxidae (Ech) | II | Serpulidae (Pol) | II |
| Cerithiopsidae (Gas) | I | Haminoeidae (Gas) | II | Ophiotrichidae (Ech) | I | Solecurtidae (Biv) | I |
| Cheirocratidae (Cru) | I | Hesionidae (Pol) | II | Ophiuridae (Ech) | II | Solemyidae (Biv) | II |
| Cidaridae (Ech) | II | Hiatellidae (Biv) | I | Paguridae (Cru) | II | Spatangidae (Ech) | I |
| Cirolanidae (Cru) | II | Hippolytidae (Cru) | I | Pandoridae (Biv) | II | Sphaeromatidae (Cru) | III |
| Clathurellidae (Gas) | I | Inachidae (Cru) | I | Parthenopidae (Cru) | I | Thiidae (Cru) | I |
| Colloniidae (Gas) | II | Janiridae (Cru) | II | Pectinariidae (Pol) | I | Tomopteridae (Pol) | II |
| Costellariidae (Gas) | I | Lasaeidae (Biv) | II | Pectinidae (Biv) | I | Toxopneustidae (Ech) | I |
| Cylichnidae (Gas) | II | Leptocheliidae (Cru) | III | Phasianellidae (Gas) | I | Triphoridae (Gas) | I |
| Cystiscidae (Gas) | II | Leptochitonidae (Ppc) | I | Philinidae (Gas) | II | Trochidae (Gas) | I |
| Diogenidae (Cru) | II | Leucosiidae (Cru) | II | Phyllodocidae (Pol) | II | Turritellidae (Gas) | I |
| Drilliidae (Gas) | I | Limidae (Biv) | I | Pilumnidae (Cru) | I | Upogebiidae (Cru) | I |
| Entalinidae (Sca) | I | Lyonsiidae (Biv) | II | Pirimelidae (Cru) | I | Xanthidae (Cru) | I |

the highest and lowest values in the data set for number of taxa, Shannon-Wiener diversity, and AMBI (Borja *et al*., 2009a). The boundary values between 'High', 'Good', 'Moderate', 'Poor' and 'Bad' EQS classes associated with each of the tested BBIs are given in Table 6.3.

**Table 6.3** Ecological Quality Status (EQS) boundary values for AMBI (Borja, Franco, & Muxika, 2004a), BENTIX (Simboura & Zenetos, 2002), BOPA and BOPA-FF (Dauvin & Ruellet, 2007), and M-AMBI (as given by the software) indices.

| | EQS Boundary Values | | | | |
|---|---|---|---|---|---|
| | AMBI | BENTIX | BOPA | BOPA-FF | M-AMBI |
| 'High' | $0.0 < x \leq 1.2$ | $6.0 \geq x \geq 4.5$ | $0.00 \leq x \leq 0.05$ | $0.00 \leq x \leq 0.05$ | $1 \geq x > 0.77$ |
| 'Good' | $1.2 < x \leq 3.3$ | $4.5 > x \geq 3.5$ | $0.05 < x \leq 0.14$ | $0.05 < x \leq 0.14$ | $0.77 \geq x > 0.53$ |
| 'Moderate' | $3.3 < x \leq 4.3$ | $3.5 > x \geq 2.5$ | $0.14 < x \leq 0.19$ | $0.14 < x \leq 0.19$ | $0.53 \geq x > 0.39$ |
| 'Poor' | $4.3 < x \leq 5.5$ | $2.5 > x \geq 2.0$ | $0.19 < x \leq 0.27$ | $0.19 < x \leq 0.27$ | $0.39 \geq x > 0.2$ |
| 'Bad' | $5.5 < x \leq 7.0$ | $2.0 > x \geq 0.0$ | $0.27 < x \leq 0.30$ | $0.27 < x \leq 0.30$ | $0.2 \geq x \geq 0.0$ |

Four-factor, permutational univariate analysis of variance (PERMANOVA) (Anderson, 2001) was based on a Euclidean similarity matrix to test the hypothesis of no differences (with level of significance [$\alpha$] set at 0.05) in values of AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices over time, with increasing distance from the tuna farms, using a model with four factors: 'Location' (Lo; 2 levels, NEF and SEF 1, fixed), 'Distance' (Di; 4 levels, Farm, Impact, 'Control 1' and 'Control 2', fixed), 'Time' (Ti; 3 levels, 2003, 2004 and 2005, random), and 'Site' (Si; 3 levels, S1, S2 and S3, random) nested within the 'Lo x Di x Ti' interaction.

Planned contrast tests between the levels of increasing distance from the tuna cages were constructed by combining sum of squares values from three separate PERMANOVAs (see Glasby, 1997) to provide for the partitioning of the factor 'Di' into three components: the test between the farm lease area, and the average of the impacted, 'Control 1' and 'Control 2' plots ('F-vs-I C1 C2'); the test between the impacted plot and the average of the 'Control 1' and 'Control 2' plots ('I-vs-C1 C2'); and the test between the 'Control 1' plot and the 'Control 2' plot ('C1-vs-C2'). The numerator/s and denominator/s used to calculate the F-ratio for the individual terms in the four-factor PERMANOVAs are given in Table 6.5. The main PERMANOVA terms of interest that assess for a spatial pattern in the influence of tuna penning activities on benthic habitat over time are the: 'F-vs-I C1 C2 x Ti'; 'I-vs-C1-C2 x Ti';

and ‘C1-vs-C2 x Ti’ interactions. The ‘Lo x F-vs-I C1 C2 x Ti’, ‘Lo x I-vs-C1 C2 x Ti’, and ‘Lo x C1-vs-C2 x Ti’ interactions indicate variability in the spatial pattern of tuna penning influence on benthic habitat over time between farming locations, while the ‘Si(Lo x F-vs-I C1 C2 x Ti)’, ‘Si(Lo x I-vs-C1 C2 x Ti)’, and ‘Si(Lo x C1-vs-C2 x Ti)’ terms indicate temporal variability at the smallest spatial scale (of a few meters).

To determine which sediment physico-chemical attribute or combination thereof, best explained the observed variation in AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices; biota and/or environment matching (BIOENV) analysis (Clarke & Gorley, 2006) was carried out, using the BEST routine with the Spearman rank correlation method, and D1 Euclidean similarity measure (Clarke & Warwick, 2001). To compare the performance of AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices with multivariate data analysis of polychaetes and amphipods that is traditionally used in aquaculture impact monitoring studies, a Principal Coordinates Analysis (PCO) (Anderson, 2003) was run using a Bray Curtis similarity matrix calculated from family abundance data that was fourth-root transformed to downweigh the highly abundant taxa (Clarke & Warwick, 2001). The relationship between the influence gradient shown by the PC1 axis scores (see Aguado-Giménez *et al*., 2015; Warwick, Clarke, & Somerfield, 2010), and the BBIs, was tested using the BEST routine with the Spearman rank correlation method, and D1 Euclidean similarity measure (Clarke & Warwick, 2001). All analyses were calculated using PRIMER v.7.0.11 (PRIMER software; Clarke & Gorley, 2006) and the PERMANOVA+ v.1.0 add-on package (Anderson *et al*., 2008).

## 6.3 Results

At the NEF farm plot: (i) the BOPA and BOPA-FF indices indicated ‘Bad’ mean EQS, the AMBI and M-AMBI indices indicated ‘Poor’ mean EQS, and the BENTIX index indicated ‘Moderate’ mean EQS, in 2003; and (ii) the BOPA-FF index indicated ‘Poor’ mean EQS, the BOPA and M-AMBI indices indicated ‘Moderate’ mean EQS, and the AMBI and BENTIX index indicated ‘Good’ mean EQS, in 2005 (Figure 6.2; Table 6.4). Furthermore, the mean EQS at the NEF impacted plot was ‘Moderate’ according to the BOPA-FF index, but ‘Good’ or ‘High’ according to the AMBI, BENTIX, BOPA

and M-AMBI indices, in 2003 (Figure 6.2; Table 6.4).

At the SEF 1 farm plot: (i) the BOPA and BOPA-FF indices indicated 'Moderate' mean EQS, the M-AMBI index indicated 'Good' mean EQS, while the AMBI and BENTIX indices indicated 'High' mean EQS, in 2003; and (ii) the BOPA and BOPA-FF indices indicated 'Bad' mean EQS, while the AMBI, BENTIX and M-AMBI indices indicated 'Poor' mean EQS, in 2005 (Figure 6.2; Table 6.4). Furthermore: (i) the BOPA index gave 'Moderate' mean EQS classification at the SEF 1 impacted plot in 2003; while (ii-a) the BOPA-FF index gave 'Poor' mean EQS classifications at the SEF 1 impacted plot in 2003 and 2004 (Figure 6.2; Table 6.4).

On the other hand, the AMBI, BENTIX and BOPA indices tended to classify the mean EQS at the NEF and SEF 1 impacted and control plots considered in general by the BOPA-FF and M-AMBI indices to be in 'Good' EQS, as 'High', during the study period (Figure 6.2; Table 6.4).

PERMANOVA indicated significant differences ($p < 0.01$) for: the interaction term 'Ti x I-vs-C1 C2' in values of the BOPA index; for 'Lo x F-vs-I C1 C2 x Ti' in values of the AMBI and M-AMBI indices ($p < 0.05$); and for 'Lo x I-vs-C1 C2 x Ti' ($p < 0.05$) and 'Lo x C1-vs-C2 x Ti' ($p < 0.001$) in values of the M-AMBI index (Table 6.5). Pair-wise tests showed that values of the AMBI index were significantly higher ($p < 0.01$) at the farm plot compared to the impacted and control plots; at the NEF in 2003 and 2005; and at the SEF 1 in 2003, 2004 and 2005. On the other hand, values of the M-AMBI index were significantly lower ($p < 0.01$): at the farm plot compared to the impacted and control plots, at both the NEF and SEF 1 in 2003 and 2005 (Table 6.5). The results of pair-wise tests showed that values of M-AMBI were significantly higher at the impacted plot compared to the control plots in 2005. Values of the AMBI and M-AMBI indices at the farm plot in 2004 and 2005, and in 2003, respectively; and of the M-AMBI index at the impacted plot in 2005; were significantly lower ($p < 0.01$) at the NEF compared to the SEF 1 (Table 6.5). Pair-wise tests showed that values of the BOPA index recorded overall at the impacted plot during the study period were significantly lower ($p < 0.01$) at the NEF compared to the SEF 1 (Table 6.5).

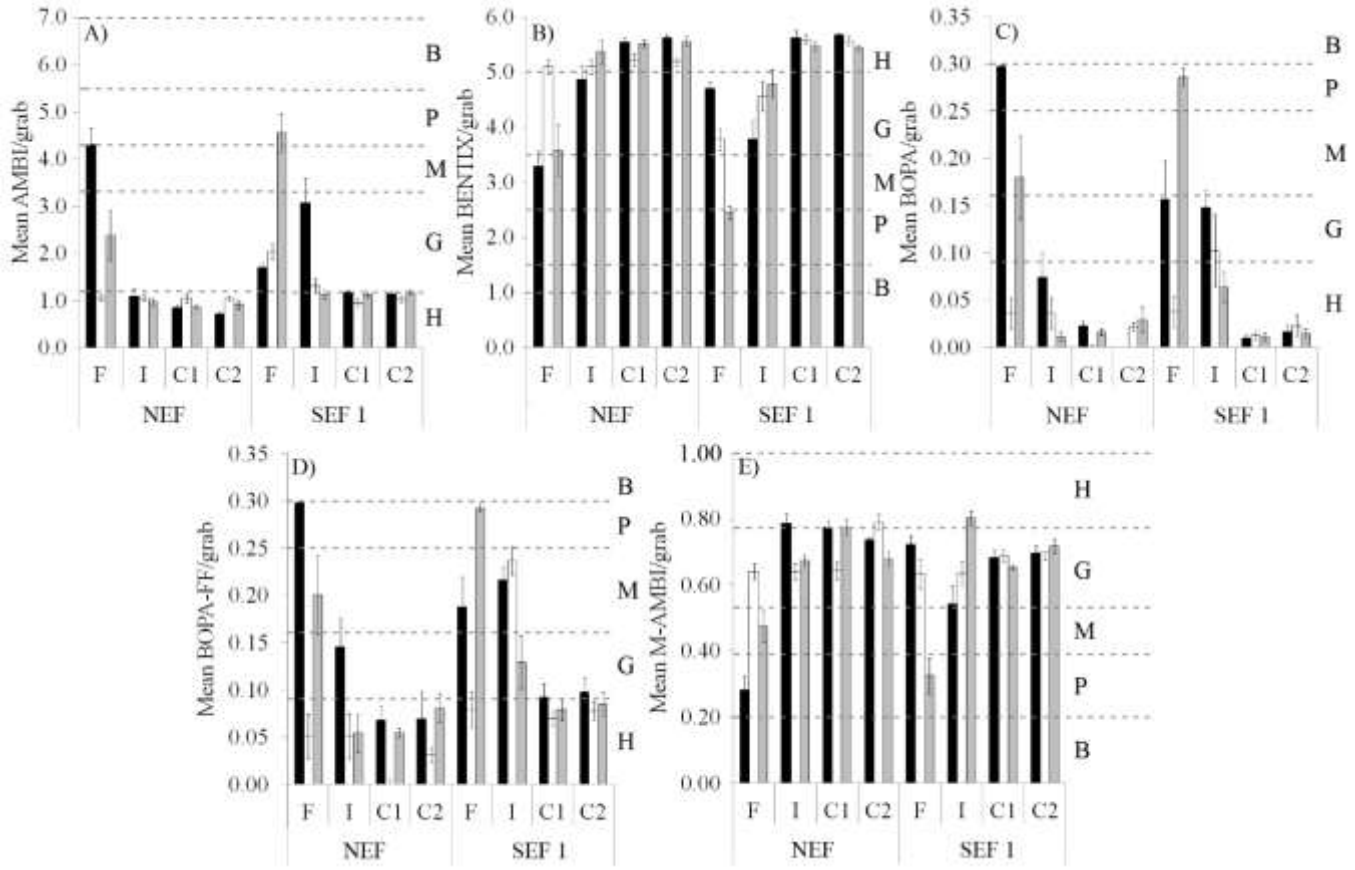


**Figure 6.2** Mean values (± SE) per grab for: (a) AMBI, (b) BENTIX, (c) BOPA, (d) BOPA-FF, and (e) M-AMBI indices recorded from farm (F), impacted (I), 'Control 1' (C1) and 'Control 2' (C2) plots in the years 2003 (black bars), 2004 (white bars) and 2005 (gray bars) at the northeastern farm (NEF) and southeastern 'Farm 1', showing 'High' (H), 'Good' (G), 'Moderate' (M), 'Poor' (P) and 'Bad' (B) EQS classifications.

**Table 6.4** Mean Ecological Quality Status (EQS) classifications for AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices recorded from farm (F), impacted (I), 'Control 1' (C1) and 'Control 2' (C2) plots in the years 2003, 2004 and 2005 at the northeastern farm (NEF) and southeastern 'Farm 1' (SEF 1). H = 'High' EQS, G = 'Good' EQS, M = 'Moderate' EQS, P = 'Poor' EQS, B = 'Bad' EQS

| NEF | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2003 | | | | 2004 | | | | 2005 | | | |
| | F | I | C1 | C2 | F | I | C1 | C2 | F | I | C1 | C2 |
| AMBI | P | H | H | H | H | H | H | H | G | H | H | H |
| BENTIX | M | H | H | H | H | H | H | H | G | H | H | H |
| BOPA | B | G | H | H | H | H | H | H | M | H | H | H |
| BOPA-FF | B | M | G | G | G | G | G | G | P | G | G | G |
| M-AMBI | P | H | H | G | G | G | G | H | M | G | H | G |
| SEF 1 | | | | | | | | | | | | |
| | 2003 | | | | 2004 | | | | 2005 | | | |
| | F | I | C1 | C2 | F | I | C1 | C2 | F | I | C1 | C2 |
| AMBI | H | G | H | H | G | H | H | H | P | H | H | H |
| BENTIX | H | G | H | H | G | H | H | H | P | H | H | H |
| BOPA | M | M | H | H | H | G | H | H | B | G | H | H |
| BOPA-FF | M | P | G | G | G | P | G | G | B | G | G | G |
| M-AMBI | G | G | G | G | G | G | G | G | P | H | G | G |

No significant differences in values of the AMBI, BENTIX, BOPA-FF, and M-AMBI indices were detected by PERMANOVA for the interaction terms 'Ti x F-vs-I C1 C2', 'Ti x I-vs-C1 C2', and 'Ti x C1-vs-C2'; and in values of the BENTIX, BOPA, and BOPA-FF indices for the interaction terms 'Lo x F-vs-I C1 C2 x Ti', 'Lo x I-vs-C1 C2 x Ti', and 'Lo x C1-vs-C2 x Ti' (Table 6.5). PERMANOVA indicated significant differences for 'Si(Lo x F-vs-I C1 C2 x Ti)' in values of the AMBI ($p < 0.01$), BENTIX ($p < 0.001$), BOPA ($p < 0.001$), BOPA-FF ($p < 0.001$), and M-AMBI ($p < 0.05$) indices; and for 'Si(Lo x I-vs-C1 C2 x Ti)' in values of the AMBI ($p < 0.01$), BOPA ($p < 0.01$), and BOPA-FF ($p < 0.01$) indices. No significant differences were detected in values of the AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices for 'Si(Lo x C1-vs-C2 x Ti)' (Table 6.5).

BEST analysis for the NEF data showed a significant correlation between: (i) the BENTIX index and PONC ($\rho = 0.398$, $p < 0.05$); (ii) the BOPA-FF index and PONC ($\rho = 0.606$, $p < 0.05$); and (iii) the M-AMBI index and a combination of MSGS and POCC ($\rho = 0.803$, $p < 0.01$), at the impacted plot; and (iv) between the AMBI index and POCC at the 'Control 1' plot ($\rho = 0.617$, $p < 0.01$) (Table 6.6).

**Table 6.5** Results of four-factor PERMANOVA for AMBI, BENTIX, BOPA, BOPA-Fish farming, and M-AMBI indices, with planned contrast tests for the factor 'Distance'. Level of significance set at 0.05. The F-ratio numerator/s and denominator/s are indicated. Df = Degrees of freedom, RES = Residual, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1'

| Source of Variation | df | AMBI | BENTIX | BOPA | BOPAFF | MAMBI | F-ratio Numerator | F-ratio Denominator |
|---|---|---|---|---|---|---|---|---|
| Location = Lo | 1 | ns | ns | ns | ns | ns | Lo and Lo x Di x Ti | Lo x Di and Lo x Ti |
| Distance = Di | 3 | ** | ** | * | ns | * | Di and Lo x Di x Ti | Lo x Di and Ti x Di |
| F-vs-I C1 C2 | 1 | ns | ns | ns | ns | ns | F-vs-I C1 C2 and Lo x F-vs-I C1 C2 x Ti | Lo x F-vs-I C1 C2 and Ti x F-vs-I C1 C2 |
| I-vs-C1 C2 | 1 | ns | ns | ns | ns | ns | I-vs-C1 C2 and Lo x I-vs-C1 C2 x Ti | Lo x I-vs-C1 C2 and Ti x I-vs-C1 C2 |
| C1-vs-C2 | 1 | ns | ns | ns | ns | ns | C1-vs-C2 and Lo x C1-vs-C2 x Ti | Lo x C1-vs-C2 and Ti x C1-vs-C4 |
| Time = Ti | 2 | ns | ns | ns | ns | ns | Ti and Lo x Di x Ti | Lo x Ti and Ti x Di |
| Lo x Di | 3 | ns | ns | ns | ns | ns | Lo x Di | Lo x Di x Ti |
| Lo x F-vs-I C1 C2 | 1 | ns | ns | ns | ns | ns | Lo x F-vs-I C1 C2 | Lo x F-vs-I C1 C2 x Ti |
| Lo x I-vs-C1 C2 | 1 | ns | ns | ** | ns | ns | Lo x I-vs-C1 C2 | Lo x I-vs-C1 C2 x Ti |
| Lo x C1-vs-C2 | 1 | ns | ns | ns | ns | ns | Lo x C1-vs-C2 | Lo x C1-vs-C2 x Ti |
| Lo x Ti | 2 | ns | ns | ns | ns | ns | Lo x Ti | Lo x Di x Ti |
| Ti x Di | 6 | ns | ns | ns | ns | ns | Ti x Di | Lo x Di x Ti |
| Ti x F-vs-I C1 C2 | 2 | ns | ns | ns | ns | ns | Ti x F-vs-I C1 C2 | Lo x F-vs-I C1 C2 x Ti |
| Ti x I-vs-C1 C2 | 2 | ns | ns | ** | ns | ns | Ti x I-vs-C1 C2 | Lo x I-vs-C1 C2 x Ti |
| Ti x C1-vs-C2 | 2 | ns | ns | ns | ns | ns | Ti x C1-vs-C2 | Lo x C1-vs-C2 x Ti |
| Lo x Di x Ti | 6 | *** | ns | * | ns | **** | Lo x Di x Ti | Si(Lo x Di x Ti) |
| Lo x F-vs-I C1 C2 x Ti | 2 | * | ns | ns | ns | * | Lo x F-vs-I C1 C2 x Ti | Si(Lo x F-vs-I C1 C2 x Ti) |
| Lo x I-vs-C1 C2 x Ti | 2 | ns | ns | ns | ns | * | Lo x I-vs-C1 C2 x Ti | Si(Lo x I-vs-C1 C2 x Ti) |
| Lo x C1-vs-C2 x Ti | 2 | ns | ns | ns | ns | *** | Lo x C1-vs-C2 x Ti | Si(Lo x C1-vs-C2 x Ti) |
| Site(Lo x Di x Ti) = Si(Lo x Di x Ti) | 48 | **** | **** | **** | **** | ** | Si(Lo x Di x Ti) | RES: Si(Lo x Di x Ti) |
| Si(Lo x F-vs-I C1 C2 x Ti) | 12 | ** | *** | *** | *** | * | Si(Lo x F-vs-I C1 C2 x Ti) | RES: Si(Lo x F-vs-I C1 C2 x Ti) |

**Table 6.5** Continued

| Source of Variation | df | AMBI | BENTIX | BOPA | BOPA-FF | M-AMBI | F-ratio Numerator | F-ratio Denominator |
|---|---|---|---|---|---|---|---|---|
| Si(Lo x I-vs-C1 C2 x Ti) | 12 | ** | ns | ** | ** | ns | Si(Lo x I-vs-C1 C2 x Ti) | RES: Si(Lo x I-vs-C1 C2 x Ti) |
| Si(Lo x C1-vs-C2 x Ti) | 24 | ns | ns | ns | ns | ns | Si(Lo x C1-vs-C2 x Ti) | RES:Si(Lo x C1-vs-C2 x Ti) |
| RES: Si(Lo x Di x Ti) | 144 | | | | | | | |
| RES: Si(Lo x F-vs-I C1 C2 x Ti) | 36 | | | | | | | |
| RES: Si(Lo x I-vs-C1 C2 x Ti) | 36 | | | | | | | |
| RES: Si(Lo x C1-vs-C2 x Ti) | 72 | | | | | | | |
| Total | 215 | | | | | | | |

| Source of Variation | | | AMBI | BENTIX | BOPA | BOPA-FF | M-AMBI | Source of Variation | | | AMBI | BENTIX | BOPA | BOPA-FF | M-AMBI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pair-wise tests -: 'Lo x Di x Ti'* | | | | | | | | | | | | | | | |
| NEF | F-vs-I C1 C2 | 2003 | > ** | - | - | - | < ** | Farm 2 | C1-vs-C2 | 2003 | - | - | - | - | ns |
| | | 2004 | ns | - | - | - | ns | | | 2004 | - | - | - | - | ns |
| | | 2005 | > ** | - | - | - | < ** | | | 2005 | - | - | - | - | > ** |
| | I-vs-C1 C2 | 2003 | - | - | - | - | ns | Farm 1 vs Farm 2 | | 2003 | ns | - | - | - | < ** |
| | | 2004 | - | - | - | - | ns | | F | 2004 | < ** | - | - | - | ns |
| | | 2005 | - | - | - | - | ns | | | 2005 | < ** | - | - | - | ns |
| | C1-vs-C2 | 2003 | - | - | - | - | ns | | | 2003 | - | - | - | - | ns |
| | | 2004 | - | - | - | - | < ** | | I | 2004 | - | - | - | - | ns |
| | | 2005 | - | - | - | - | < ** | | | 2005 | - | - | - | - | < ** |
| SEF 1 | F-vs-I C1 C2 | 2003 | > ** | - | - | - | < ** | | | 2003 | - | - | - | - | > ** |
| | | 2004 | > ** | - | - | - | ns | | C1 | 2004 | - | - | - | - | ns |
| | | 2005 | > ** | - | - | - | < ** | | | 2005 | - | - | - | - | > ** |
| | I-vs-C1 C2 | 2003 | - | - | - | - | ns | | | 2003 | - | - | - | - | ns |
| | | 2004 | - | - | - | - | ns | | C2 | 2004 | - | - | - | - | > * |
| | | 2005 | - | - | - | - | < ** | | | 2005 | - | - | - | - | ns |

**Table 6.5** Continued

| Source of Variation | | AMBI | BENTIX | BOPA | BOPA-FF | M-AMBI |
|---|---|---|---|---|---|---|
| *Pair-wise tests -: 'Lo x Di x Ti'* | | | | | | |
| NEF | F-vs-I C1 C2 | - | - | - | - | - |
| | I-vs-C1 C2 | - | - | ns | - | - |
| | C1-vs-C2 | - | - | - | - | - |
| SEF 1 | F-vs-I C1 C2 | - | - | - | - | - |
| | I-vs-C1 C2 | - | - | ns | - | - |
| | C1-vs-C2 | - | - | - | - | - |
| NEF vs SEF 1 | F | - | - | - | - | - |
| | I | - | - | < ** | - | - |
| | C1 | - | - | - | - | - |
| | C2 | - | - | - | - | - |

BEST analysis for the SEF 1 data showed significant correlation between: (i) the BOPA-FF index and MSGS at the impacted plot ($\rho = 0.673$, $p < 0.05$), and (ii) between the M-AMBI index and MSGS at the 'Control 2' plot ($\rho = 0.558$, $p < 0.05$) (Table 6.6). BEST analysis did not indicate a significant correlation between the AMBI, BENTIX, BOPA, BOPA-FF and M-AMBI indices, and sediment physico-chemical variables below the cages of either tuna farm, or at the reference site located 2 km away from the SEF 1, nor did it indicate a significant correlation between the BOPA index and sediment physico-chemical variables, at either tuna farm (Table 6.6).

The results of PCO analysis indicated that the PC1 axis explained 39.6% and 32.2% of the total variation in polychaete and amphipod family abundance data (Figure 6.3). BEST analysis showed a significant, positive correlation, between values of the AMBI ($\rho = 0.419$, $p < 0.001$), BENTIX ($\rho = 0.369$, $p < 0.01$), BOPA ($\rho = 0.374$, $p < 0.01$), BOPA-FF ($\rho = 0.384$, $p < 0.01$), and M-AMBI ($\rho = 0.406$, $p < 0.01$) indices, and the influence gradient shown by the PC1 axis of the amphipod faunal group (Figure 6.3). Values of the AMBI and M-AMBI indices showed the strongest correlation with the disturbance gradient shown by the amphipod faunal group, followed by values of the BOPA-FF and BOPA indices, and of the BENTIX index. Values of the AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices indicated 'Good' and 'High' EQS at the lower end of this influence gradient; while values of the BOPA and BOPA-FF index also classified some 'low impact' samples as 'Moderate' and 'Poor'. At the upper end of the influence gradient, values of the BOPA and BOPA-FF indices indicated 'Bad' EQS, while values of the M-AMBI index and of the AMBI and BENTIX indices respectively indicated 'Poor' EQS, and 'Moderate' and 'Poor' EQS (Figure 6.3).

Values of the M-AMBI index were significantly and positively correlated with the PC1 axis of the polychaete faunal group ($\rho = 0.234$, $p < 0.05$), but assigned 'Poor', 'Moderate' and 'Good' EQS, and 'Good' and 'High' EQS, respectively at the lower and upper ends of the influence gradient. There was no significant correlation between the influence gradient shown by the PC1 axis of the polychaete faunal group, and values of the AMBI, BENTIX, BOPA, and BOPA-FF indices (Figure 6.3).

**Table 6.6** BEST results showing the explanatory variable or combination thereof that best explains the observed variation in AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI indices at the northeastern farm (NEF) and southeastern 'Farm 1' (SEF 1) farm, impacted and control plots. ρ-value = Spearman rank correlation coefficient, Best Exp Var = Best Explanatory Variable, MSGS = mean sediment grain size, POCC = percent organic carbon content, PONC = percent organic nitrogen content, ns = not significant, * = $p < 0.05$, ** = $p < 001$

| | | AMBI | | BENTIX | | BOPA | |
|---|---|---|---|---|---|---|---|
| | | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| NEF | Farm | 0.074, ns | MSGS | 0.076, ns | MSGS | 0.050, ns | MSGS |
| | Impact | 0.433, ns | POCC, PONC | 0.398, * | PONC | 0.394, ns | PONC |
| | 'Control 1' | 0.617, ** | POCC | 0.307, ns | MSGS, PONC | -0.081, ns | POCC |
| | 'Control 2' | 0.125, ns | MSGS | 0.162, ns | POCC | 0.520, ns | MSGS, POCC, PONC |
| SEF 1 | Farm | 0.231, ns | MSGS | 0.277, ns | MSGS | 0.034, ns | PONC |
| | Impact | 0.020, ns | POCC | 0.315, ns | POCC | 0.012, ns | MSGS |
| | 'Control 1' | 0.002, ns | MSGS | 0.151, ns | POCC | 0.149, ns | PONC |
| | 'Control 2' | 0.034, ns | MSGS, POCC | 0.203, ns | MSGS, POCC | 0.389, ns | MSGS, POCC, PONC |
| | | BOPA-FF | | M-AMBI | | | |
| | | ρ-value | Best Exp Var | ρ-value | Best Exp Var | | |
| NEF | Farm | 0.162 , ns | MSGS | 0.101, ns | PONC | | |
| | Impact | 0.606, * | PONC | 0.803, ** | MSGS, POCC | | |
| | 'Control 1' | -0.104, ns | POCC | 0.161, ns | POCC | | |
| | 'Control 2' | 0.384, ns | PONC | 0.356, ns | MSGS, POCC, ONC | | |
| SEF 1 | Farm | 0.045, ns | PONC | 0.229, ns | PONC | | |
| | Impact | 0.673, * | MSGS | 0.501, ns | MSGS | | |
| | 'Control 1' | 0.210, ns | PONC | 0.314, ns | MSGS, POCC | | |
| | 'Control 2' | 0.044, ns | MSGS, POCC, PONC | 0.558, * | MSGS | | |

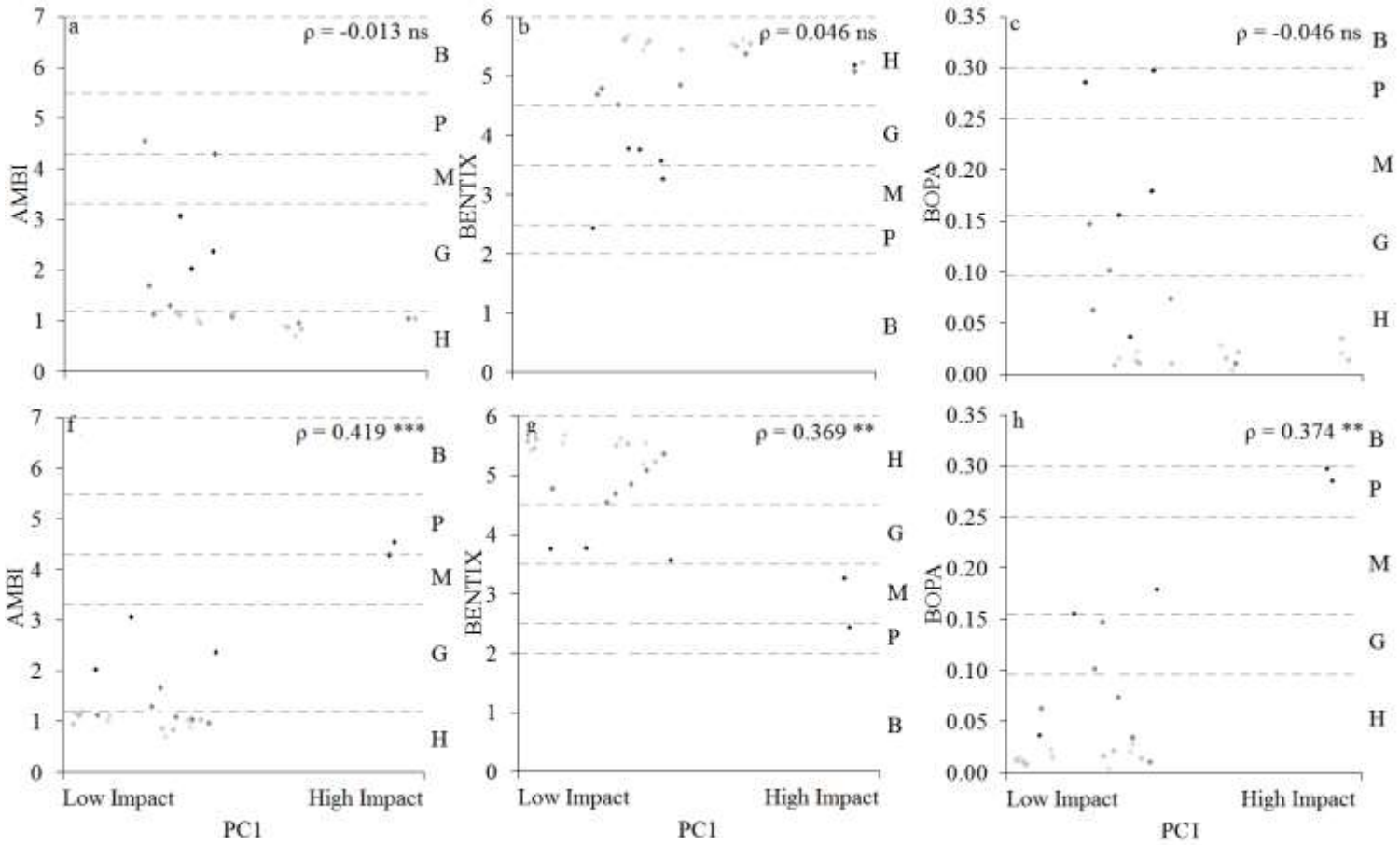


**Figure 6.3** Scatter plots showing mean values of (a, f) AMBI, (b, g) BENTIX, (c, h) BOPA, (d, i) BOPA-FF, and (e, j) M-AMBI plotted against the disturbance gradient (PC1) described by the multivariate polychaete (a-e) and amphipod (f-j) family abundance data recorded from the northeastern and southeastern 'Farm 1' farm (black), impacted (dark grey) and control (light grey) plots in the period 2003 to 2005, showing the EQS classification. ρ = Spearman rank correlation coefficient, ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$, H = 'High' EQS, G = 'Good' EQS, M = 'Moderate' EQS, P = 'Poor' EQS, B = 'Bad' EQS

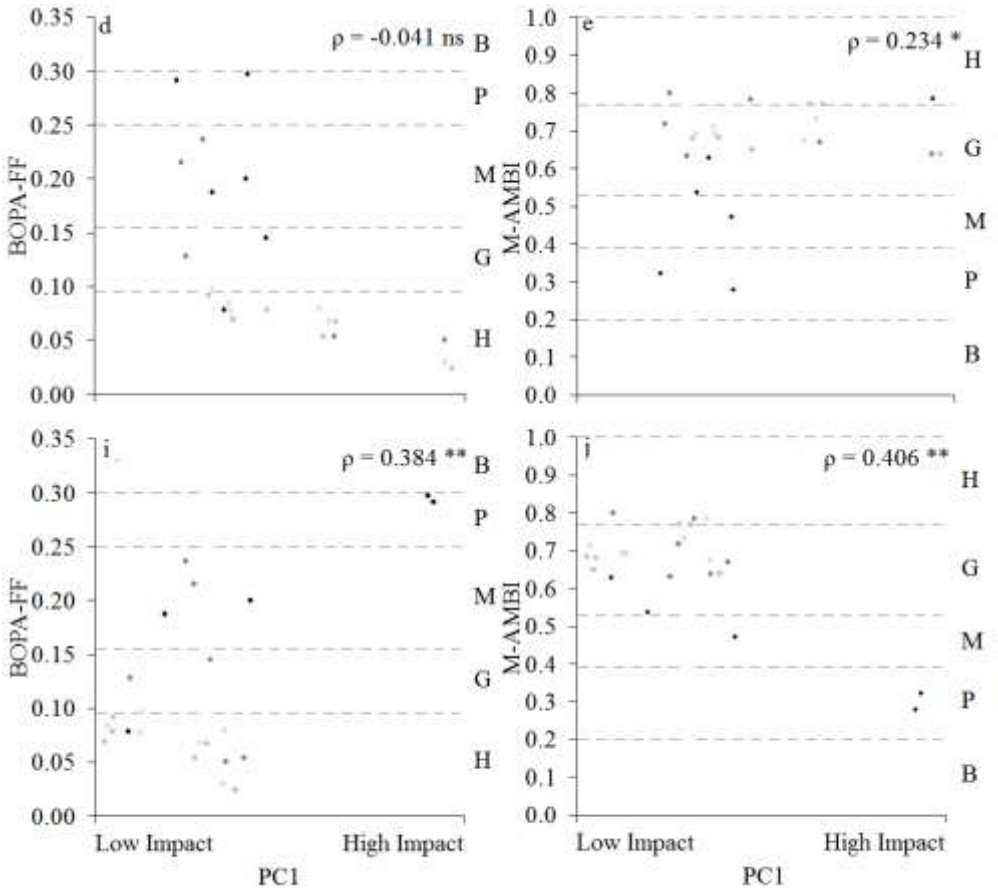


**Figure 6.3** Continued

## 6.4 Discussion

The BBIs assessed in the present study; i.e. AMBI, BENTIX, BOPA, BOPA-FF, and M-AMBI; showed a spatial pattern of stressed macroinvertebrate assemblages that was characterized by a high impact radius on the seabed directly below the fish cages, and improved ecological quality at reference sites located 1 km and 2 km away from the tuna farms. Other workers have similarly used the AMBI (e.g. Aguado-Giménez *et al*., 2007; Forchino *et al*., 2011; Katsiaras *et al*. 2010; Muxika *et al*. 2005; Tomassetti *et al*., 2009), BENTIX (e.g. Aguado-Giménez *et al*., 2007; Katsiaras *et al*., 2010), BOPA (e.g. Aguado-Giménez *et al*., 2015; Jahani *et al*., 2012), BOPA-FF (e.g. Aguado-Giménez *et al*., 2015; Mangion *et al*., 2017), and M-AMBI (e.g. Forchino *et al*., 2011; Tomasetti *et al*., 2009) indices to describe spatial patterns in benthic assemblages under the influence of fish farming activities in the Mediterranean.

For the same farms considered in the present study, Mangion *et al*. (unpublished data) reported accumulation of fish-bones in the sediment directly below the cages, which was higher at the SEF 1 compared to the NEF (see Section 5.3.1). No significant difference was detected in MSGS and POCC in sediment with increasing distance from the farms, while significantly higher levels of PONC in sediment were recorded at a distance of 100 m from the fish cages compared to reference areas located 1-2 km away, at both tuna farms (Mangion *et al*., unpublished data) (see Section 5.3.1). Keeley *et al*. (2012) noted that some physico-chemical sediment features do not reflect early stages of organic enrichment at sites located in a high energy environment, while BBIs including AMBI and M-AMBI readily detect significant changes in benthic assemblages under these conditions. On the other hand, Sampaio, Rodrigues, and Quintino (2011) suggested that biotic indices, including those based on species tolerance/sensitivity to organic enrichment, may not be effective in detecting changes in macrofaunal assemblages at mild enrichment levels. Previous studies reported significant correlation between values of AMBI, BENTIX, BOPA, and M-AMBI indices, and mean grain size and organic matter content of the sediment (Bouchet & Sauriau, 2008). Other studies reported significant correlation between values of the AMBI index and levels of polycyclic aromatic hydrocarbon, total organic carbon (Muniz, Venturini, Pires-Vanin, Tommasi, & Borja, 2005), total hydrocarbons (Muxika *et al*., 2005), and total organic matter content (Borja *et al*., 2009c) in sediments. The

present results showed significant correlation at the impacted and control sites between values of the AMBI and BENTIX indices and the POCC and PONC of the sediment, respectively, and between values of the BOPA-FF and M-AMBI indices, and combinations of MSGS and PONC, and MSGS and POCC, respectively. However, no significant correlation between the BBIs and sediment attributes was recorded below the cages of either tuna farm. Subida *et al*. (2012) previously noted that BBIs are not correlated to organic carbon at levels lower than 3%, as was the case in the present study (See Section 5.3.1). Other workers also did not find a significant correlation between values of the BENTIX index and sediment organic carbon content (Simboura & Reizopoulou, 2007; Simboura & Zenetos, 2002). It is worth noting that other sediment physico-chemical attributes, such as the total free sulfide content, which is a good chemical indicator of fish farm environmental impacts (see Aguado-Giménez *et al*., 2015), are not taken into account in the present study; values of these may be correlated with values of BBIs for benthic assemblage data within the seabed area directly below the sea cages.

Brauko, de Souza, Muniz, de Camargo, and da Cunha Lana (2015) reported low agreement among values of indices when using these to describe spatial variation of macrofaunal assemblages, with only values of AMBI being significantly correlated with sediment physico–chemical attributes. AMBI is particularly useful for assessment of the influence of aquaculture activities, since the main indicators of fish farm impacts as defined by Edgar *et al*. (2010) - i.e. the bivalve/mollusc ratio and the frequency of Capitellids - are included in AMBI, while values of sediment redox potential are a strong predictor of AMBI (Forchino *et al*., 2011). Values of the AMBI, BENTIX, and BOPA indices are based on the classification of taxa into EGs and show significant inter-correlation (Dauvin *et al*., 2007). While BBIs, in general, rank sampling stations in the same way, lack of agreement in EQS assessment is widely reported in the literature, especially at stations having low values for ecological quality (e.g. Aguado-Giménez *et al*., 2007; Bouchet & Sauriau, 2008; Dauvin *et al*., 2007; Keeley *et al*., 2012; Simboura & Argyrou, 2010; Simonini *et al*., 2009; Spagnolo *et al*., 2014), such that assignment of EQS will vary depending on the BBI applied (Dauvin *et al*., 2007). Values of the AMBI and BENTIX indices do not result in classification of the same EQS (e.g. Aguado-Giménez *et al*., 2007; Dauvin *et al*., 2007) due to the different importance each index gives to the different EGs, and the different threshold values

and ecological quality ratios set among categories of EQS (Simboura & Reizopoulou, 2007). Simboura (2004) suggested that the AMBI index may be more suitable for use in assessing communities in the Atlantic and in estuarine areas, that are characterized by a low biodiversity; i.e. few species and high densities, such that discrimination among the various EGs is required to reflect a change in ecological quality. On the other hand, the BENTIX index may be more suitable for Mediterranean coastal waters that are characterised by a high biodiversity and by evenly distributed species abundances. Simboura (2004) noted that the AMBI index has lower discriminating power compared to the BENTIX index, while the latter tends to give a lower EQS assignment (classifying 'Poor') from the two indices. The robustness of the AMBI index is decreased when the number of individuals or/and species is very low (Borja & Muxika, 2005), which causes problems in assessment of intermediate benthic ecological states (Salvo *et al*., 2017). Simboura and Reizopoulou (2008) concluded that the BENTIX index performs better than the AMBI index in Eastern Mediterranean coastal sites, while the AMBI index has been applied with success elsewhere in the Mediterranean Sea (e.g. Aguado-Giménez *et al*., 2007; Borja *et al*., 2009c; Forchino *et al*., 2011; Muxika *et al*., 2005). Forchino *et al*. (2011) previously used the AMBI index to show low ecological quality at Italian farms growing sea bream and sea bass, while other studies showed that the spatial pattern in variation of attributes of the macrofaunal assemblages with distance from sea cages may be sufficiently described by the AMBI index (Borja *et al*., 2009b), or by a combination of the AMBI and BENTIX indices (Aguado-Giménez *et al*., 2007).

In the present study, the AMBI index at times appeared more sensitive to the ecological influence of tuna penning activities on benthic habitat compared to the BENTIX index; the former showing lower EQS within the footprint of the NEF during the study period. The positive correlation with the disturbance gradient, as indicated by the multivariate amphipod family abundance data, was stronger for the AMBI index compared to the BENTIX index; while values of the AMBI index differed significantly over time under the influence of tuna penning activities, values of the BENTIX index did not. On the other hand, no disagreement in the assignment of EQS was noted for sites having better ecological quality, with values of the AMBI and BENTIX index classifying samples in 'Good' or 'High' EQS with consistency; this contrasts with previous results obtained by Simboura and Reizopoulou (2007), who noted that the AMBI index tends

to classify most sites that are classified as having 'Moderate' and often 'High' EQS sites according to BENTIX, as 'Good'.

Problems are encountered with the AMBI index when classification of taxa into EGs by the AMBI list is not appropriate for local conditions (e.g. Teixeira *et al*., 2012). The difficulty of assigning taxa into EGs is reduced in the BENTIX index since it involves only three groups compared to the AMBI index, which involves five (Simboura, 2004; Simboura & Zenetos, 2002). On the other hand, the BENTIX index is less accurate compared to the AMBI index since it classifies all species into just three EGs, resulting in overlap of two different intermediate responses into one EQS category (Dauvin *et al*., 2007). It is appropriate that the EG assignment for taxa will be regionally validated for the different environmental conditions when applying them internationally (e.g. Aguado-Giménez *et al*. 2007; Borja *et al*., 2011; Borja & Muxika 2005; Keeley *et al*., 2012; Teixeira *et al*., 2012). There may also be misclassification of taxa in the BENTIX index, since the data base is mainly derived from a taxon list for the Eastern Mediterranean (Tomassetti *et al*., 2016). Assignment of taxa into tolerant and indifferent groups may be more subjective compared to assignment of taxa into sensitive and first order opportunistic species (Dauvin *et al*., 2010). Since the ecological strategy of species in the Maltese Islands is poorly defined, the use of family level data is recommended (Forde *et al*., 2013). Family level data was previously used with success when using the AMBI (Tweedley, Warwick, Clarke & Potter, 2014), BOPA (Aguado-Giménez *et al*., 2015), and M-AMBI (Forde *et al*., 2013) indices, and the Benthic Quality Index (Dimitriou *et al*., 2012). In the present study, 84 families novel to the AMBI list (version June 2017) and not previously assigned into EGs by Forde *et al*. (2013), were assigned EGs using the median value of all taxa within the parent family after Forde *et al*. (2013), and validated using Best Professional Judgment (see Teixeira *et al*., 2012) to calculate the BBIs.

The BOPA index requires less taxonomic effort than the AMBI and BENTIX indices, hence decreasing the time for identification and the probability of identification errors (Dauvin & Ruellet, 2007). However, indices based on a limited number of taxa may not be suitable for application in different geographical regions because they are affected by endemism (Keeley *et al*., 2012). There is high correlation between the AMBI and BOPA indices since the latter's classification of opportunistic polychaetes

is based on the former's list of EGs (Subida *et al*., 2012), but these two indices do not give the same EQS assessment since they are based on different ecological models of tolerance (Bouchet & Sauriau, 2008): to organic matter input in the AMBI index (Borja *et al*., 2000) and to hydrocarbons in the BOPA index (Dauvin & Ruellet, 2007; Gomez-Gesteira & Dauvin, 2000). De-la-Ossa-Carretero *et al*. (2009) advice caution in the application of the BOPA index to oligotrophic waters that are characterised by low macrofaunal abundance and a patchy distribution of species, as occurs in the Mediterranean Sea. Riera and De-la-Ossa-Carreter (2014) indicate that the BOPA index appears to give reliable responses only in strongly impacted areas, and weak responses in areas affected by fish farming in the Atlantic Ocean. Similarly, Wang *et al*. (2017) noted that the AMBI and M-AMBI indices are more suitable for assessing the EQS than the BOPA index when the disturbance is slight, such as in the case of bivalve aquaculture. Aguado-Giménez *et al*. (2015) showed that the BOPA index does not describe the influence gradient resulting from aquaculture that is detected by traditional multivariate data analysis when the latter is used in impact assessment studies. While the applicability of the BOPA index is improved by inclusion of polychaete families known to be local indicator taxa that are tolerant to fish farming impacts, misclassification still occurs, especially at the lower part of the influence gradient (Aguado-Giménez *et al*., 2015). Such erroneous categorisation by the BOPA index occurs when the amphipod abundance is low and tolerant polychaete families are present in reference areas (Aguado-Giménez *et al*., 2015). BOPA may also underestimate the EQS (Aguado-Giménez *et al*., 2015; De-la-Ossa-Carretero *et al*., 2009) when the total abundance of polychaetes and amphipods is very high and decreases the frequency of amphipods (Aguado-Giménez *et al*., 2015). Jahani *et al*. (2012) previously reported that the BOPA index indicated 'Bad' EQS below fish cages, where the abundance, biomass and diversity were low compared to reference conditions. In the present study, the performance of the BOPA index improved when polychaete indicator taxa tolerant to fish farm pollution (see Martinez-Garcia *et al*., 2013) were used to calculate the index; such results are similar to those obtained by Aguado-Giménez *et al*. (2015). In general, both the BOPA and BOPA-FF indices indicated lower ecological quality for the seabed area directly below the cages of the two farms compared to the AMBI and BENTIX indices, while only the BOPA-FF index signaled decreased ecological quality some 100 m away from the cages of the two farms, as a result of the tuna penning activities. However, the correlation between

values of the BOPA index and of the BOPA-FF index, and the disturbance gradient described by the multivariate amphipod family abundance data, were weaker compared to the correlation between values of the AMBI and M-AMBI indices and the same amphipod disturbance gradient.

Furthermore, the BOPA-FF index showed no significant difference along the spatio-temporal pattern of influence of tuna penning on the seabed due to high variation at the smallest spatial scale (i.e. 100 m's). Small scale variation characteristic of soft bottom assemblages (e.g. Chapman *et al*., 2010; Morrisey, Howitt, Underwood, & Stark, 1992; Stark *et al*., 2003), is particularly high for stressed assemblages (e.g. Stark *et al*., 2003; Warwick & Clarke, 1993) at fish farm sites compared to control areas (Fernandez-Gonzalez *et al*., 2013). Small scale variation reduces the power of statistical tests made using data from hierarchical nested designs to detect observed differences (see Morrisey *et al*., 1992) when the small scale variation is larger than at the higher spatial scales (e.g. Anderson *et al*., 2005; Chapman *et al*., 2010; Fernandez-Gonzalez *et al*., 2013; Fraschetti *et al*., 2005), as this confounds changes in ecological quality recorded by the biotic indices (Brauko *et al*., 2015), leading to difficulty in the interpretation of results.

Subida *et al*. (2012) noted that the potential of diversity indices to respond monotonically to an increase in levels of sediment organic matter in the Mediterranean Sea is lower than that of indices based on indicator taxa, due to the oligotrophic nature of the sea. Aguado-Giménez *et al*. (2007) noted that Shannon-Wiener diversity may not be sufficient to describe spatial patterns in macrobenthic assemblages under the influence of fish farming activities. For the same tuna farms considered in the present study, both values of the Shannon-Wiener diversity of polychaetes and amphipods (Mangion *et al*., unpublished data) (see Section 5.3.1), and of the AMBI index were significantly low for the seabed area immediately below the sea cages. Furthermore, values of the Shannon-Wiener diversity index for amphipods signaled a significant influence of tuna penning activities on benthic habitat that extended up to 100 m away from the sea cages (Mangion *et al*., unpublished data) (see Section 5.3.1); on the other hand the AMBI index did not. These observations are in concordance with the results from other studies which indicated that values of the AMBI and Shannon-Wiener diversity indices do not give similar patterns when assessing the influence of fish

farming activities on marine benthic assemblages (e.g. Callier *et al*., 2008; Spagnolo *et al*., 2014).

Subida *et al*. (2012) noted that the M-AMBI index has higher correlation with the Shannon-Weiner diversity than it does with the AMBI index, which lowers its correlation with the spatial pattern in sediment organic loading compared to the AMBI index. The present results showed that the M-AMBI index was significantly correlated with the MSGS and POCC of the sediment 100 m away from the cages of the NEF, while values of the AMBI index did not show a significant correlation with sediment attributes in the immediate vicinity of the farms; this does not corroborate the results obtained by Subida *et al*. (2012). Some workers do not recommend the use of the M-AMBI index because of the weight it gives to species richness and diversity, which are dependent on sample size, seasonal variation, and habitat type (Simboura & Argyrou, 2010; Subida *et al*., 2012; but see Borja, Mader, Muxika, Rodríguez, & Bald, 2008). The AMBI and M-AMBI indices do not always give the same EQS assessment (e.g. Sivaraj, Murugesan, Muthuvelu, Vivekanandan, & Vijayalakshmi, 2014). Simonini *et al*. (2009) reported good performance of the M-AMBI index compared to the BENTIX and AMBI indices when applied in the Adriatic Sea. Khedhri, Afli, and Aleya (2017) reported good agreement between the AMBI, BENTIX, and M-AMBI indices when using them for assigning EQS in Mediterranean lagoons. The present results indicate that while both the AMBI and M-AMBI indices showed significantly low ecological quality below the cages at both farms during the study period, values of the M-AMBI index at times indicated lower EQS below the tuna cages compared to values of the AMBI index. The calculation of values of the M-AMBI index requires use of reference values for the number of taxa, Shannon-Wiener diversity index, and the AMBI index, which respectively represent the best and worst ecological statuses of a study area (Muxika *et al*., 2007). The identification of reference conditions for a study area is challenging when no real reference sites or historical data exist (Paganelli, Forni, Marchini, Mazziotti, & Occhipinti-Ambrogi, 2011). The reference values used to calculate values of the M-AMBI index in the present study were selected from the data set (which included reference sites located 2 km away from the farms) using the default option in the M-AMBI software, such that the results obtained by the M-AMBI index must be interpreted with caution, and since the EQS classification may change with use of different reference data. In the present study, the M-AMBI index tended to

classify control sites considered by the AMBI index to be in 'High' EQS, as 'Good'. This observation contrasts with the local application of the AMBI and M-AMBI indices by MEPA (2013), which indicated that the M-AMBI index classifies most stations considered by the AMBI index to be in 'Good' EQS, as 'High'.

Other workers concluded that, since multivariate analysis of physico-chemical and macrobenthic data is accurate and statistically validated, they may be more appropriate than values of benthic indices for use in environmental monitoring of fish farming activities (Aguado-Giménez *et al*., 2015). Similarly, Quintino *et al*. (2012) showed that multivariate abundance data, together with taxon richness and total abundance, are more effective than biotic indices in detecting benthic habitat changes resulting from oyster farming. The same taxonomic effort is required while avoiding possible errors in assigning taxa into EGs (Aguado-Giménez *et al*., 2007). The analysis of full species composition datasets provides a more reliable picture of environmental disturbance since biotic indices may cause information loss and hence impair the diagnostic capability (Sampaio *et al*., 2011). In the present study, the BBIs performed well compared to traditional multivariate data analyses used to detect ecological impact at fish farms; they showed significant correlation with the influence axis described by the amphipod family abundance, and correctly classified most sites at the lower and upper ends of the disturbance gradient into 'Good' and 'High' EQS, and into 'Poor' and 'Moderate' EQS, respectively. For the same farms included in the present study, the influence gradient described by the multivariate polychaete family abundance, and containing taxa with different sensitivity/tolerance levels along the organic enrichment gradient (see Giangrande *et al*., 2005; Martinez-Garcia *et al*., 2013), was not well defined (see Section 5.3.2). Furthermore, only values of the M-AMBI index were significantly correlated to the disturbance gradient described by the polychaete assemblage, while the relationship between the M-AMBI index and the latter influence axis was upside-down, similar to results obtained by Aguado-Giménez *et al*. (2015). The multivariate amphipod family abundance, containing solely taxa sensitive to pollution (Dauvin, 1987, 1998) (with the exception of the genus *Jassa* [Dauvin & Ruellet, 2007]), were more useful (see Section 5.3.2). Values of the AMBI and M-AMBI indices showed better correlation with the disturbance gradient described by the amphipod faunal group compared to the BOPA-FF and BOPA

indices, while the BENTIX index showed lower correlation compared to the AMBI, M-AMBI, BOPA-FF, and BOPA indices.

The AMBI and BENTIX indices tended to indicate better ecological quality for the seabed area occupied by the cages compared to the BOPA, BOPA-FF, and M-AMBI indices.'High' ecological quality at reference areas was not classified with consistancy: the BOPA-FF and M-AMBI indices indicated 'Good' EQS, while the AMBI, BENTIX and BOPA indices indicated 'High' EQS, at control plots during the study period. The influence of tuna penning activities at the impacted plot of both tuna farms was indicated only by values of the BOPA and BOPA-FF index; values of the AMBI, BENTIX, and M-AMBI indices did not signal decreased ecological quality resulting from tuna penning activities, at this distance. Intermediate benthic states may be misclassified (Simboura, 2004). Disagreement in EQS assignment between different indices may result in wrong management decisions that may have serious envrionmental consequences (Aguado-Giménez *et al*., 2015). Blanchet *et al*. (2008) confirmed the limitations of biotic indices and the poor decisions which they can induce. A study by Khedhri *et al*. (2017) reported that the indicating power of biotic indices was reduced by harmful algal blooms that increased deposition of organic matter and promoted a higher polychaete abundance, consequently classifying sampled stations at 'High' EQS. Workers indicate that a multi-metric approach provides a better assessment compared to use of single indices (e.g. Lavesque, Blanchet, & de Montaudouin, 2009; Purnomo Putro, 2011), while others recommend the use of an average score of various indices (H', AMBI, BENTIX, BOPA, and M-AMBI) that accounts for different types of information over the use of single and multi-metric indices (Bouchet & Sauriau, 2008). Aguado-Giménez *et al*. (2015) stressed the need for benthic indices to be designed for specific activities, and to be validated at regional levels, due to the adaptive capacities of macroinvertebrates to their environment.

The BBIs tested in the present study gave values that significantly correlated with the pattern of ecological disturbance caused by tuna penning activities as described by the traditional multivariate analyses of amphipod family abundance data, and correctly classified sites at the lower and upper ends of the influence gradient into 'Good'/'High' EQS and 'Moderate'/'Poor' EQS, respectively. Of the tested BBIs, only the BOPA

index showed no significant relationship with sediment physico-chemical attributes. The performance of the BOPA index improved when using polychaete families tolerant to fish farm pollution. The AMBI, BENTIX, BOPA-FF, and M-AMBI indices depended on MSGS, and sediment POCC and/or PONC at the impacted and control sites, while no significant relationship with sediment physico-chemical attributes below the sea cages was detected at either of the two tuna farms. Some disagreement in EQS classification occurred, particularly at the lower end of the influence gradient. In general, the BENTIX index tended to over-estimate the EQS below tuna cages compared to the AMBI index, while the M-AMBI index tended to indicate lower ecological quality below the tuna cages compared to the AMBI index. Both the BOPA and BOPA-FF indices indicated lower ecological quality for the seabed area occupied by the cages compared to the AMBI, BENTIX, and M-AMBI indices. Only the BOPA and BOPA-FF indices indicated decreased ecological quality within a 100 m radius from the tuna cages, while the AMBI, BENTIX, and M-AMBI indices did not signal decreased ecological quality resulting from the tuna penning activities at this distance.

In conclusion, in assessments of the environmental influence of tuna penning on benthic habitat, the BOPA-FF, AMBI, and M-AMBI indices appear more suitable for use in the Maltese Island compared to the BENTIX and BOPA indices. Of the tested BBIs, the BOPA-FF index appears more sensitive to fish farm pollution compared to the AMBI, BENTIX, BOPA, and M-AMBI indices, while the AMBI and M-AMBI indices showed the strongest correlation with the fish farm disturbance gradient described by the multivariate amphipod family abundance data. These observations suggest that the use of a complementary set of indices such as the BOPA-FF and M-AMBI indices may be more appropriate to estimate the EQS at fish farm sites in the Maltese Islands due to the disagreement in EQS assignment. Furthermore, in assessments of the environmental influence of tuna penning on benthic habitat, BBIs should be used in conjunction with the multivariate analyses of sediment physico-chemical and macroinvertebrate taxon abundance data to avoid wrong management decisions based on erroneous EQS categorization.

# CHAPTER 7

# TEMPORAL PATTERNS IN BENTHIC ASSEMBLAGES UNDER THE INFLUENCE OF TUNA PENNING

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## 7.1 Introduction

Anthropogenic disturbance of the marine environment may be classified as either short-term ('pulse') or as continuous ('press') disturbance (Glasby & Underwood, 1996). Pollution caused by fish farming activities is more similar to natural, pulse-type, organic enrichment events, compared to other anthropogenic disturbances, and results from the repeated fish farm production cycles that alternate with fallow periods (Macleod *et al*., 2007). Recovery of sediments from pollution by fish farm wastes is faster compared to recovery from anthropogenic disturbance of soft bottoms by industrial pollutants, since the former results from pollution by waste that consists mainly of uneaten feed and faeces, and is easily degraded (e.g. Karakassis *et al*., 1999; Shin, Lam, Wu, Qian, & Cheung, 2008). A general review of recovery rates and patterns for coastal and estuarine habitats from different types of antrhopogenic disturbances is available in Borja *et al*. (2010a).

The temporal pattern in benthic assemblages recovering from organic enrichment is similar to that observed along a spatial gradient of organic enrichment as described by the Pearson-Rosenberg (1978) model (e.g. Lu & Wu, 2000; Macleod *et al*., 2007, 2008), but may interrupted in the early stages of succession due to secondary disturbances (e.g. Karakassis *et al*., 1999; Sanz-Lazáro & Marin, 2006). Pollution intolerant, suspension-feeding, crustacean infauna; such as amphipods (Dauvin, 1987, 1998); are the first to disappear from azoic sediment conditions under high levels of organic enrichment, and become replaced by a large population of deposit-feeding opportunists (Nilsson & Rosenberg, 1994; Pearson & Rosenberg, 1978) such as capitellid polychaetes (Borja *et al*., 2000). The pioneer community undergoes a series of successions to reach a final mature status (Rosenberg, Agrenius, Hellman, Nilsson, & Norling, 2002) following recovery from organic enrichment, which should be similar to the community at reference areas that are free from pollution (Karakassis *et al*., 1999).

The temporal pattern in deposition of organic matter on the seabed and the resultant benthic influence originating from fish farming activities arises from temporal variability in the fish farm production cycle, since this leads to variation in the amount of feed entering the water column, in the consumption of feed by the farmed species,

and in the resulting organic enrichment (e.g. Jansen *et al*., 2016; Kutti *et al*., 2007a; Tomassetti *et al*., 2016). The periodic abandonment of fish farm sites in winter (fallowing) that halts feed input serves to minimise the marine environmental impacts of fish farming activities by allowing for some recovery of the benthos to take place between production cycles (e.g. Macleod *et al*., 2006; Zhulay *et al*., 2015). Some recovery of the seabed during winter also takes place via the resuspension of organic matter from the sediment by strong, underwater sea currents (Karakassis *et al*., 1998). Previous studies addressed the recovery of benthic communities from organic enrichment following fish farm abatement (e.g. Borja *et al*., 2010a; Brooks, Stierns, & Backman, 2004; Karakassis *et al*., 1999; Keeley *et al*., 2014, 2015; Lu & Wu, 1998; Macleod, Crawford, & Moltschaniwsky, 2004; Macleod *et al*., 2006, 2007, 2008; Mangion *et al*., 2014; Pereira *et al*., 2004; Pohle, Frost, & Findlay, 2001; Salvo *et al*., 2017; Sanz-Lázaro & Marin, 2006; Zhulay *et al*., 2015). The period taken for complete benthic recovery below fish cages following cessation of fish farming activities varies from months to several years, depending on the initial level of impact, length of fallow period, and farm location (Macleod *et al*. 2006, 2007). The long recovery times reported for benthic assemblages following fish farm abatement (e.g. Aguado-Giménez *et al*., 2007; Borja *et al*., 2010a; Brooks *et al*., 2004; Keeley *et al*., 2014, 2015; Macleod *et al*., 2007; Pohle *et al*., 2001; Salvo *et al*., 2017) indicate that the benthic community may return to the pre-fallowed state as soon as production is resumed (Pereira *et al*., 2004). When insufficient time is given for the benthic habitat to recover between production cycles, fish farming activities may lead to cumulative, 'press' disturbance and to significantly damaged sediment ecological function, rendering fish farming unviable (Macleod *et al*., 2007). While previous studies have addressed the use of potential indicators in long-term environmental monitoring programs at fish farms in different geographical regions (e.g. Edgar *et al*., 2010; Riera *et al*., 2012; Tomassetti *et al*., 2016), as far as the present author is aware, no previous work has examined the temporal pattern of disturbance in benthic assemblages during the repeated use of a site for aquaculture.

Workers recommend the use of indicator taxon indices for use in long-term monitoring programmes rather than diversity indices, since the classification of taxa into EGs is less sensitive to seasonal and interannual variation than diversity (Kröncke & Reiss, 2010). Previous studies have assessed the applicability of different indicator taxon

indices developed for monitoring in relation to the Water Framework Directive (WFD, 2000/60/EC), to classify coastal water bodies into 'High', 'Good', 'Moderate', 'Poor', or 'Bad' Ecological Quality Status (EQS) classes; and for use in long-term monitoring programs of different types of environmental disturbances (e.g. Borja *et al*., 2009a; Dauvin & Ruellet, 2007; Muxika *et al*., 2007; Sanz-Lazáro & Marin, 2006; Simboura, Papathanassiou, & Sakellariou, 2007; Simboura, Zenetos, & Pancucci-Papadopoulou, 2014). Other studies examined the suitability of various indicator taxon indices in evaluation of benthic ecological influence of fish farming activities (e.g. Aguado-Giménez *et al*., 2007; Borja *et al*., 2009c; Edgar *et al*., 2010; Karakassis *et al*., 2013; Katsiaras *et al*., 2010; Muxika *et al*., 2005) and compared their performance with multivariate analyses used in aquaculture environmental impact monitoring studies (Aguado-Giménez *et al*., 2007, 2015) that traditionally use polychaetes (e.g. Aguado-Giménez *et al*., 2015; Martinez-Garcia *et al*., 2013; Nobrega-Silva *et al*., 2016) and amphipods (e.g. Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011; Mangion *et al*., 2017) as benthic biological indicators. While different indicator taxon indices usually reflect the same general pattern of ecological quality and are significantly inter-correlated (e.g. Dauvin *et al*., 2007, 2012; Karakassis *et al*., 2013; Subida *et al*., 2012), there is often disagreement in the EQS assigned to individual sampling sites (e.g. Aguado-Giménez *et al*., 2007; Bouchet & Sauriau, 2008; Dauvin *et al*., 2007; Labrune *et al*., 2012; Simboura, 2004; Simboura & Argyrou, 2010; Wu *et al*., 2013). Such disagreement in assignemnt of an EQS to a given site renders the inclusion of sediment physico-chemical variables necessary in the assessment (Tomassetti *et al*., 2016), while the use of a combination of various indices over single or multi-metric indices (Bouchet & Sauriau, 2008) used in conjunction with the more powerful multivariate analyses of macroinvertebrate data, is recommended to avoid information loss and wrong management decisions based on erroneous EQS categorisations (Aguado-Giménez *et al*., 2015).

The environmental influence of Atlantic Blue-fin Tuna (ABT) farming on sediment physico-chemical attributes and macroinvertebrate assemblages has been previously described in the Mediterranean (Aguado *et al*., 2004; Aguado-Giménez *et al*., 2006; Mangion *et al*., 2014, 2017; Mangion, Borg, & Sanchez-Jerez, 2018; Marin *et al*., 2007; Matijević *et al*., 2006, 2008; Vezzulli *et al*., 2008; Vita & Marin 2007; Vita *et*

*al*., 2004a). However, it seems that no studies that assess the long-term, temporal pattern, in benthic assemblages at sites used repeatedly for ABT aquaculture have been carried out to date. In the present study, the indicator taxon indices BOPA-FF (Aguado-Giménez *et al*., 2015) and M-AMBI (Borja *et al*., 2004b; Muxika *et al*., 2007) are used with sediment physico-chemical data; namely the w/w feed-fish bone content (FFBC) - which represents the amount of uneaten feed-fish that decomposed on the seabed -; mean sediment grain size (MSGS); percent organic carbon content (POCC), and percent organic nitrogen content (PONC); and in conjunction with the more powerful multivariate analyses of polychaete and amphipod assemblages, to assess for a temporal pattern in the marine environmental disturbance during ten years of ABT farming. The experimental design included data collected from three different ABT farms and six reference areas before initiation of tuna penning and after initiation of the activity, at six-monthly or annual intervals. The main aim of the present work was to determine whether the tuna penning activities resulted in a 'pulse' or 'press' disturbance at sites used repeatedly for ABT aquaculture.

## 7.2 Material and methods

### 7.2.1 Study sites and sampling

The three tuna farms included in the present study were located circa 1 km off the northeastern to southeastern coast of the Maltese Islands (Figure 7.1) where the seabed consisted of 'bare sand' habitat and the water depth was some 42 m to 53 m. One farm was located off the northeastern coast, and had eight tuna cages with a maximum total annual capacity of 2500 t (ICCAT, 2011). The other two farms were smaller, having a maximum total annual capacity of 1500 t each (ICCAT, 2011), and located off the southeastern coast. The two farms were separated by a distance of 1 km. One farm (southeastern 'Farm 1', 'SEF 1') had three tuna cages, while the other (southeastern 'Farm 2', 'SEF 2') had four tuna cages (ICCAT, 2011). Tuna penning operations started in summer in 2001 at the northeastern farm ('NEF') and at the SEF 2, and in 2003 at the SEF 1. The three farms utilized cages having a diameter of 50 m and a height of 25 m. The farm lease areas were: 350 m x 500 m (NEF), 550 m x 550 m (SEF 1), and 300 m x 500 m (SEF 2). The tuna were stocked at a density of circa 2-4 kg/$m^3$, and fed the equivalent of 3-4 % of the fish biomass per day, over two feeding sessions

(tuna farm managers, personal communication, January 14, 2015). The feed consisted of whole bait fish; namely mackerel, sardines, prawn, and squid; with a ratio of feed that is converted into tuna biomass of circa 10-15:1 (tuna farm managers, personal communication, January 14, 2015).

Samples were collected from three plots that had the same seabed type, and from a similar water depth: (a) the 'impact' plot, i.e. the seabed area immediately below the fish cages; (ii) the 'Control 1' plot, located circa 1 km away from the cages; and (iii) the 'Control 2' plot, located circa 2 km away from the cages (Figure 7.1). This sampling design was replicated at each of the three farms. A number of sampling sites, separated by some 100 meters, were allotted to each plot: four at the NEF and the SEF 1, since these farms had four cages, and three at the SEF 2, since this farm had three cages; such that a total of 33 sampling sites were included in the sampling design. The latitude/longitude coordinates and depth of the sampling sites are shown in Table 7.1.

The cost-benefit ratio of the environmental monitoring programme for the proximate southeastern farms was maximised by using joint environmental monitoring and by reducing the sampling effort to common control sites using only the 'Control 2' plots from June'06 thereafter (see Figure 7.1).

Tuna penning activities were initiated during summer in 2001 at the NEF and the SEF 2, and in 2003 at the SEF 1. Sampling of the 'bare sand' habitat was carried out as part of an environmental monitoring programme as required by the local environmental and planning authority. Sediment samples for benthic macrofaunal studies were collected in November 2000 and March 2001 at the NEF, in October 2002 at the SEF 1, and in June 2001 at the SEF 2, before farming commenced; and in November'01, April'02, Janunary'03, April'03, November'03, March'04, November'04, November'05, April'06, June'07, May'08, and April'09 at the NEF; in October'03, October'04, October'05, June'06, June'07, June'08, and June'09 at the SEF 1; and in June'02, June'03, June'04, June'05, June'06, June'07, June'08, and June'09 at the SEF 2, following initiation of the tuna penning activities.

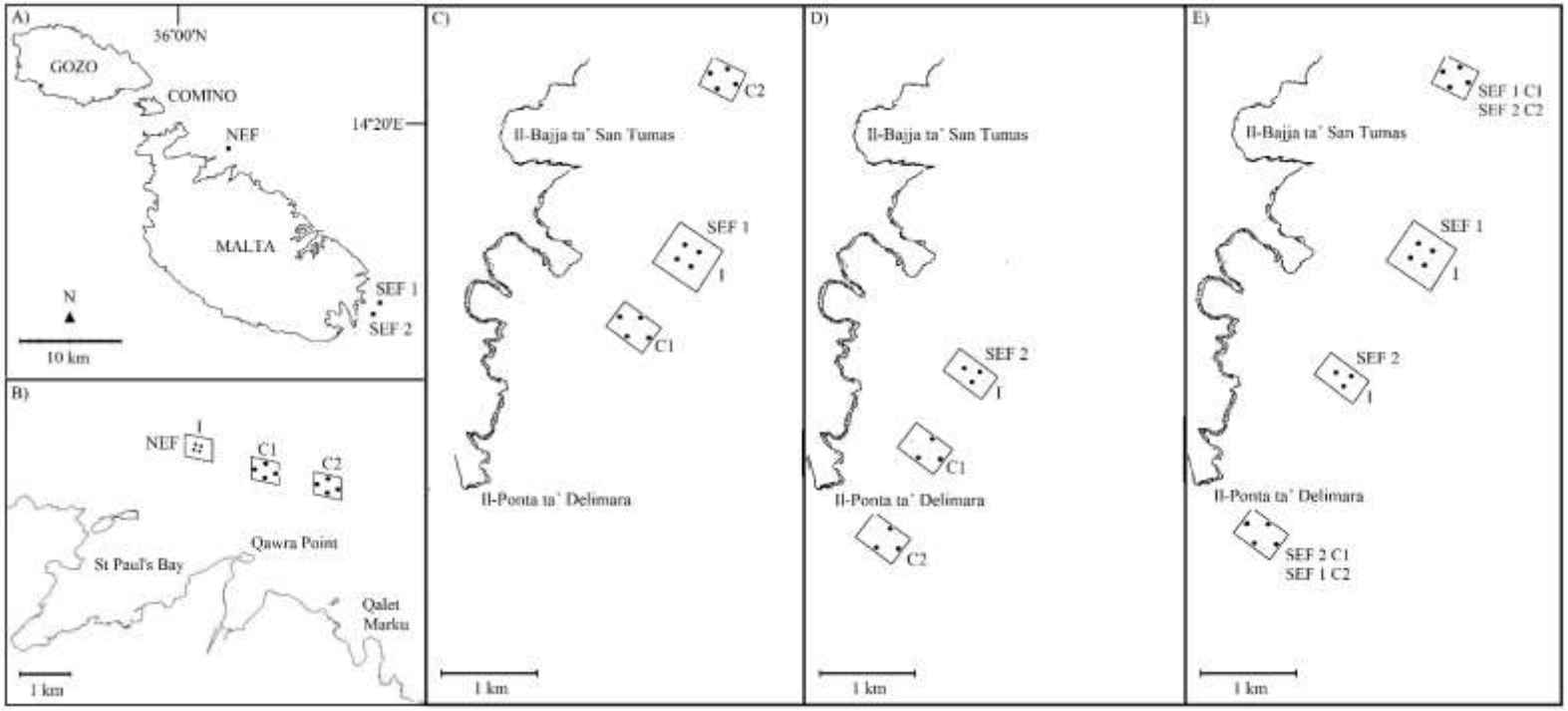


**Figure 7.1** Map of the Maltese Islands showing the locations of: (a) the three tuna farms; (b) the three sampling plots at the northeastern farm (NEF); (c) the three sampling plots at southeastern 'Farm 1' (SEF 1); and (d) the three sampling plots at southeastern 'Farm 2' (SEF 2); from where samples for benthic macrofaunal and sediment physico-chemical studies were collected. The environmental monitoring programme was revised in June'06 to incorporate common control sites for the two adjacent southeastern tuna farms (e). I = impacted plot, C1 = 'Control 1' plot, C2 = 'Control 2' plot

**Table 7.1** Latitude/longitude coordinates and depth of the control sites shown in Figure 7.1b-d. The impacted plot was centered on: N35º 58.66'/E14º 25.16' at the NEF; N35º 50.17'/E14º 35.11' at the SEF 1; and N35 º 49.85'/E14º 34.67' at the SEF 2. Samples from the impacted plot were collected from the seabed area directly below the tuna-pens.

| | | NEF | | SEF 1 | | SEF 2 | |
|---|---|---|---|---|---|---|---|
| Plot | Site | Latitude/Longitude | Depth (m) | Latitude/Longitude | Depth (m) | Latitude/Longitude | Depth (m) |
| 'Control 1' | S1 | N35º 58.32/E14º 26.72 | 50 | N35º 50.18/E14º 34.79 | 51 | N35º 49.55/E14º 34.41 | 47 |
| | S2 | N35º 58.18/E14º 26.85 | 50 | N35º 50.10/E14º 34.85 | 51 | N35º 49.35/E14º 34.48 | 47 |
| | S3 | N35º 58.12/E14º 26.72 | 48 | N35º 50.11/E14º 34.70 | 46 | N35º 49.36/E14º 34.28 | 46 |
| | S4 | N35º 58.45/E14º 26.16 | 52 | N35º 50.21/E14º 34.68 | 42 | - | - |
| 'Control 2' | S1 | N35º 58.51/E14º 26.33 | 52 | N35º 51.58/E14º 35.42 | 47 | N35º 49.02/E14º 34.15 | 48 |
| | S2 | N35º 58.38/E14º 26.47 | 52 | N35º 51.50/E14º 35.48 | 47 | N35º 48.83/E14º 34.22 | 49 |
| | S3 | N35º 58.32/E14º 26.33 | 51 | N35º 51.49/E14º 35.37 | 45 | N35º 48.87/E14º 34.00 | 48 |
| | S4 | N35º 58.26/E14º 26.59 | 50 | N35º 51.58/E14º 35.33 | 43 | - | - |

Samples for sediment physico-chemical studies were collected in the same periods, with the exception of 'before' samples from the NEF, which were collected only in March'01. Three replicate grab samples for benthic macrofaunal studies, and one grab sample for sediment physico-chemical studies, were collected at each of the 33 sampling sites using a 0.1 $m^2$ van Veen grab. The collected samples were sieved using a 0.5 mm mesh on board the vessel, and the material retained by the sieve was preserved in 10 % formalin saline.

In the laboratory, the samples were washed on a 0.5 mm mesh to remove the formal saline and the retained fauna was sorted for macroinvertebrates. Specimens were identified to the family level (see Karakassis & Hatziyanni, 2000; Olsgard & Somerfield, 2000) and enumerated to obtain estimates of the number of individuals per grab sample. For sediment physico-chemical studies, sub-samples for the determination of percent organic carbon content (POCC), percent organic nitrogen content (PONC), and weight/weight feed-fish bone content (FFBC) were frozen at -20 °C for later analysis, while another sub-sample was oven dried for granulometric analysis. Analysis of the sediment to determine the FFBC was carried out for samples collected from below the fish cages by micro-sorting of the sediment. POCC in the sediment was determined by wet oxidation using a chromic acid-sulfuric acid mixture and titration of the evolved carbon dioxide (see Walkley & Black, 1934). PONC in the sediment was determined by the Kjeldhal method, i.e. by digestion in concentrated sulfuric acid containing a copper sulfate catalyst, addition of excess strong alkali, and condensation of the ammonia given off for titration (see Holme & McIntyre, 1984). Measurement of mean sediment grain size (MSGS) was carried out according to Buchanan (1984) (see Holme & McIntyre, 1984).

Unpublished data on sea current direction and velocity, at water depths ranging from 1 to 10 m, were available from surveys undertaken once every 3 months at the northeastern and southeastern farm sites, during the period 2010-2017, using the Lagrange method (see Bennett, 2006).

### 7.2.2 Data analyses

Since the sampling dates varied among the three ABT farms, data analysis was carried out separately for each farm. Values of the BOPA-FF index were calculated using BOPA-FF = log {($f_P$ / $f_A$ +1) +1}; where '$f_P$' is the frequency of polychaetes tolerant to organic enrichment resulting from fish farming activities, as identified by Martinez-Garcia *et al*. (2013) (see Aguado-Giménez *et al*., 2015), and '$f_A$' is the frequency of amphipod individuals that are sensitive to organic enrichment, excluding the genus *Jassa* (Dauvin & Ruellet, 2007). Boundary values between 'High' ($0.00 \leq x \leq 0.05$), 'Good' ($0.05 < x \leq 0.14$), 'Moderate' ($0.14 x \leq 0.19$), 'Poor' ($0.19 < x \leq 0.27$), and 'Bad' ($0.27 < x \leq 0.30$) EQS classes for BOPA-FF were used as given in Dauvin and Ruellet (2007). Values of the M-AMBI index were calculated using reference values obtained by using the highest and lowest values in the data set for number of taxa, Shannon-Wiener diversity, and the AMBI index (Borja *et al*., 2009a). The boundary values between 'High' ($1 \geq x > 0.77$), 'Good' ($0.77 \geq x > 0.53$), 'Moderate' ($0.53 \geq x > 0.39$), 'Poor' ($0.39 \geq x > 0.2$), and 'Bad' ($0.2 \geq x \geq 0.0$) EQS classes for the M-AMBI index are as provided by the software.

Three-factor, univariate, permutational analysis of variance (PERMANOVA) (Anderson, 2001) was run (with level of significance [α] set at 0.05) on a Euclidean similarity matrix, to test the hypothesis of no differences in values of the BOPA-FF and M-AMBI indices under the influence of tuna penning activities over time, using a model with two orthogonal factors: 'Time' (Ti; 14 levels at the NEF, 8 levels at the SEF 1, and 9 levels at the SEF 2; fixed) and 'Plot' (Pl; 3 levels, with a fixed component, Impact, and two random components, 'Control 1' and 'Control 2'), and a factor 'Site' (Si; 4 levels at the NEF and SEF 1, and 3 levels at the SEF 2; random) nested within the 'Pl x Ti' interaction. Separate two-factor univariate PERMANOVA was carried out (with α set at 0.05) on the data using a Euclidean similarity matrix to test the hypothesis of no differences in the MSGS, and POCC and PONC of the sediment, using a similar experimental design, with levels of 'Si' treated as replicates.

An asymmetrical design was applied to the factor 'Pl' since there were two control plots for each impacted plot (Underwood, 1992, 1994). Asymmetrical PERMANOVAs were calculated by combining sum of squares values from three separate

PERMANOVAs (see Glasby, 1997) to provide for the partitioning of the factor 'Pl' into two components: the contrast test between the impacted plot and the average of the two control plots ('Impact-vs-Control') ('Im-vs-Co'), and the random variability among the two control plots ('Co'). The numerator/s and denominator/s used in the calculation of the F-ratio for the individual terms in the two-factor and three-factor asymmetrical PERMANOVAs are respectively given in Tables 7.1 and 7.2. The main PERMANOVA term of interest that assesses for an influence of tuna penning activities on benthic habitat over time is the 'Im-vs-Co x Ti' interaction, while the 'Co x Ti' interaction indicates spatial variation (at the scale of 1 km) between control areas over time. The 'Si(Im-vs-Co x Ti)' and 'Si(Co x Ti)' interaction terms indicate temporal variability at the smallest spatial scale (100's of meters). When the asymmetrical PERMANOVA indicated significant differences for the 'Im-vs-Co x Ti' interaction term, *a posteriori* pair-wise comparisons were carried out (with α set at 0.05) to investigate differences between sampling times at impacted and control plots, while 'Im-vs-Co' comparisons were carried out per sampling time (with α set at 0.05) using post-hoc asymmetrical PERMANOVAs for the factor 'Pl'.

To test the hypothesis of no significant differences (α set at 0.05) in polychaete and amphipod family abundance under the influence of tuna penning activities over time, three-factor, asymmetrical, permutational, multivariate ANOVA (PERMANOVA) (Anderson, 2001; McArdle & Anderson, 2001) was carried out using the Bray Curtis similarity matrix calculated from family abundance data that was fourth-root transformed to downweigh the highly abundant taxa (Clarke & Warwick, 2001). A permutational multivariate dispersion test (PERMDISP) (Anderson, 2004, 2006) was then used to calculate differences (α set at 0.05) in within-group dispersion using the sample distance to the centroid of each of the factors. In both PERMANOVA and PERMDISP tests, a total of 9999 unrestricted permutations of raw data were used, with α set at 0.05. Principal coordinate analysis (PCO) (Anderson, 2003) was carried out and the results were plotted to show differences in polychaete and amphipod family abundances between impacted, 'Control 1', and 'Control 2' plots over time. The two-factor model was used to test for significant differences in sediment physico-chemical attributes using similar multivariate analyses on a D1 Euclidean similarity matrix calculated from environmental data that was normalised to homogenize the different units (Clarke & Warwick, 2001). The three most important taxa contributing to the

dissimilarity in polychaete and amphipod assemblages between impacted and control plots per sampling time were identified using the similarity percentages of species contributions (SIMPER) method (Clarke & Warwick, 2001), and post hoc three-factor univariate, asymmetrical PERMANOVA was carried out (with α set at 0.05) using the abundance data for polychaete and amphipod taxa that contributed to high dissimilarities between impacted and control plots. To determine which sediment physico-chemical attribute or combination thereof, best explained the observed variation in values of the BOPA-FF and the M-AMBI indices, and the family abundance of polychaetes and amphipods, the BEST routine analysis of the biota and/or environment matching (BIOENV) test (Clarke & Gorley, 2006) was carried out using the Spearman rank correlation method and D1 Euclidean similarity measure at the level of the factors (since the number of replicates at the level of the two-way interaction term 'Pl x Ti' was too low). PERMANOVA, PERMDISP, PCO, SIMPER, and BEST analyses were carried out using PRIMER v.7.0.11 (PRIMER software; Clarke & Gorley, 2006) and the PERMANOVA+ v.1.0 add-on package (Anderson *et al*., 2008).

## 7.3 Results

### 7.3.1 Univariate data analyses

#### 7.3.1(i) Sediment physico-chemical attributes

FFBC in the sediment below the tuna cages at the NEF was elevated in November'01, and varied from 0.0 $\pm$ 2.7% (April'03 & June'07) to 1.8 $\pm$ 16.2% (November'01) throughout the study period (Figure 7.2). Below SEF 1 tuna cages, FFBC was elevated in October'03 and varied from 0.0 $\pm$ 2.8% (June'08) to 1.6 $\pm$ 13.4% (October'03). The level of FFBC recorded below SEF 2 tuna cages was low throughout the study period (0.0% $\pm$ 2.7% to 0.6% $\pm$ 4.8%) (Figure 7.2).

PERMANOVA indicated a significant difference ($p < 0.0001$) for 'Im-vs-Co x Ti' (and no significant difference for 'Co x Ti') in the level of POCC at the NEF and SEF 2 (Table 7.2). *A posteriori* comparisons showed no significant difference in POCC at the NEF and SEF 2 between impacted and control plots before tuna penning

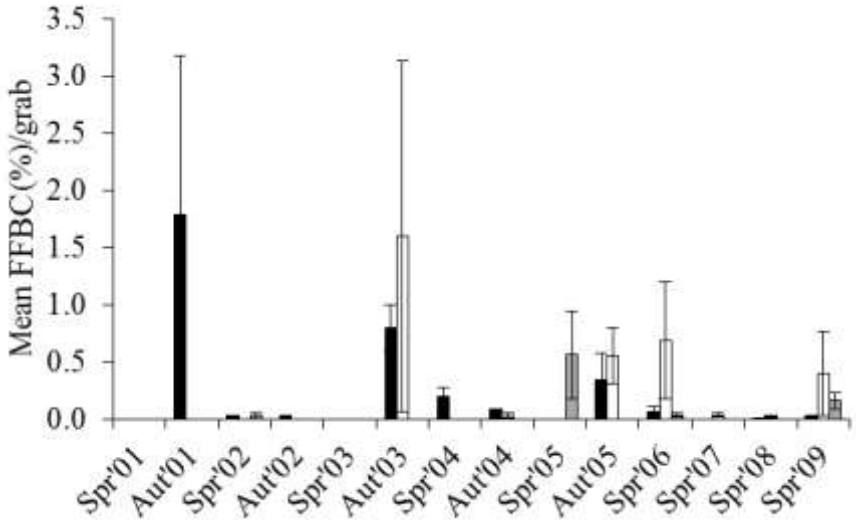


**Figure 7.2** Mean values (± SE) per grab of w/w percent feed-fish bone content (FFBC) in the sediment collected from below the cages at the northeastern farm (NEF) (black bars), southeastern 'Farm 1' (SEF 1) (white bars), and southeastern 'Farm 2' (SEF 2) (grey bars). Tuna penning activities started durig summer in 2001 at the NEF and the SEF 2, and in 2003 at the SEF 1. Spr = spring, Aut = autumn

commenced (March'01) (Figure 7.3; Appendix 1). Following initiation of the tuna penning activities, POCC increased significantly at the NEF impacted plot in November'01, April'02, April'03, November'03, November'04, November'05, May'08, and April'09 ($p < 0.01$ except $p_{\text{April'02}} < 0.05$ & $p_{\text{November'05}} < 0.001$) compared with levels recorded in March'01. The level of POCC recorded at the NEF impacted plot in November'01 was significantly higher compared with values of the same attribute recorded at the NEF control plots in the same period ($p < 0.001$) and at the NEF impacted plot in subsequent sampling periods (April'02, January'03, & November'03-April'09) ($p < 0.05$ except $p_{\text{June'07, May'08}} < 0.05$). No significant differences in POCC between the NEF impacted and control plots during the rest of the study period (April'02-April'06, May'08, & April'09) were detected by the statistical analysis (Figure 7.3; Appendix 1).

At the SEF 2, levels of POCC in sediment decreased significantly ($p < 0.01$ except $p_{\text{June'06}} < 0.001$) at the impacted plot in June'06-June'08 compared with levels recorded before tuna penning commenced (June'01). Levels of POCC in sediment recorded at the SEF 2 impacted plot in June'07 and June'08 were significantly higher ($p < 0.001$) compared with values of the same attribute recorded at the SEF 2 control plots in the same period, but significantly lower compared with values of the same attribute

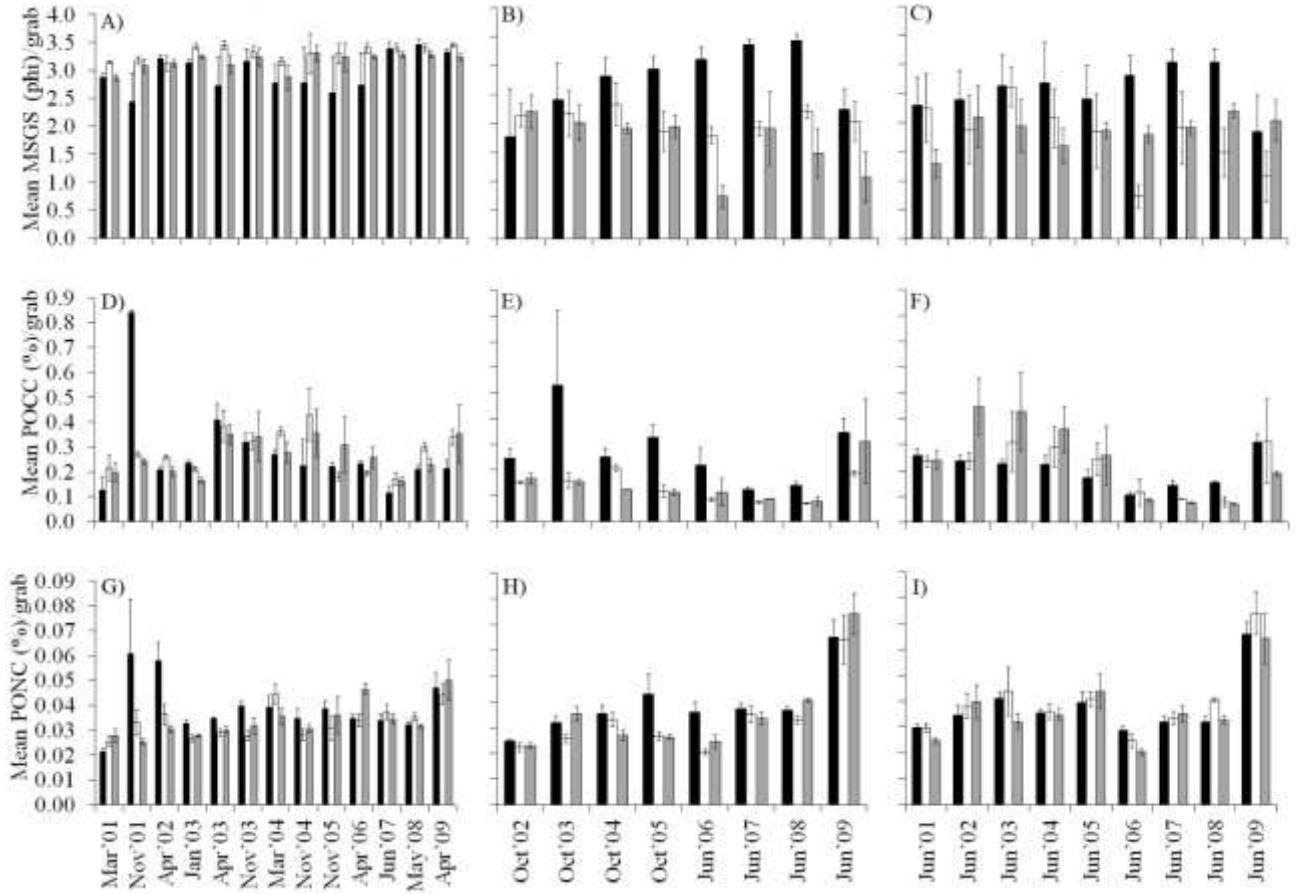


**Figure 7.3** Mean values (± SE) per grab of: (a, b, c) mean sediment grain size (MSGS) (phi), (d, e, f) percent organic carbon content (POCC) in sediment, and (g, h, i) percent organic nitrogen content (PONC) in sediment recorded from impacted (black bars), 'Control 1' (white bars) and 'Control 2' (grey bars) plots at the (a, d, g) northeastern farm, (b, e, h) southeastern 'Farm 1', and (c, f, i) southeastern 'Farm 2'

**Table 7.2** Results of two-factor univariate, asymmetrical PERMANOVA for mean sediment grain size (MSGS) (phi), percent organic carbon content (POCC) in sediment, and percent organic nitrogen content (PONC) in sediment recorded from the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1) and southeastern 'Farm 2' (SEF 2). The F-ratio numerator/s and denominator/s are also shown. The level of significance was set at 0.05. Df = degrees of freedom, RES = Residual, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, F-Ratio Num = F-Ratio numerator, F-Ratio Den = F-Ratio denominator

| | | NEF | | | | SEF 1 | | |
|---|---|---|---|---|---|---|---|---|
| Source of Variation | df | MSGS | POCC | PONC | df | MSGS | POCC | PONC |
| Impact-vs-Control = Im-vs-Co | 1 | ** | ns | ns | 1 | ** | ns | ns |
| Control = Co | 1 | *** | ns | ns | 1 | ns | ns | ns |
| Time = Ti | 12 | ns | ns | ns | 7 | ns | ns | ns |
| Im-vs-Co x Ti | 12 | ns | **** | ns | 7 | ns | ns | ns |
| Co x Ti | 12 | ns | ns | ns | 7 | ns | ns | ns |
| RES: Im-vs-Co x Ti | 39 | | | | 17 | | | |
| RES: Co x Ti | 78 | | | | 32 | | | |
| Total | 155 | | | | 72 | | | |

| | | SEF 2 | | | | |
|---|---|---|---|---|---|---|
| Source of Variation | df | MSGS | POCC | PONC | F-Ratio Num | F-Ratio Denom |
| Impact-vs-Control = Im-vs-Co | 1 | *** | ns | ns | Im-vs-Co | Im-vs-Co x Ti |
| Control = Co | 1 | ns | ns | ns | Co | Co x Ti |
| Time = Ti | 8 | ns | * | **** | Ti | Im-vs-Co x Ti + Co x Ti |
| Im-vs-Co x Ti | 8 | ns | **** | ns | Im-vs-Co x Ti | RES: Im-vs-Co x Ti |
| Co x Ti | 8 | ns | ns | ns | Co x Ti | RES: Co x Ti |
| RES: Im-vs-Co x Ti | 27 | | | | | |
| RES: Co x Ti | 54 | | | | | |
| Total | 107 | | | | | |

recorded at the SEF 2 impacted plot in June'02 ($p < 0.05$), June'03 ($p_{\text{June'07}} < 0.05$ & $p_{\text{June'08}} < 0.01$) and June'09 ($p < 0.01$). No significant differences in POCC between SEF 2 impacted and control plots during the rest of the study period were detected by the statistical analysis (June'01-June'06 & June'09) (Figure 7.3; Appendix 1).

The low MSGS recorded in November'01, April'03, March'04, November'05 and April'06, and the high levels of PONC recorded in November'01 and April'09 at the NEF impacted plot compared with the NEF impacted plot before tuna penning commenced (March'01), and compared to the NEF control plots in the same periods (Figure 7.3), were not statistically significant (PERMANOVA, 'Im-vs-Co x Ti'; Table 7.2). Similarly, the observed general increase in MSGS at the two SEF impacted plots

during the study period, and the peak in levels of POCC and PONC in sediment at the SEF 1 impacted plot in October'03 and October'05, and at the impacted and control plots of both SEFs in June'09 (Figure 7.3), were not statistically significant (PERMANOVA, 'Im-vs-Co x Ti'; Table 7.2).

### 7.3.1(ii) Macroinvertebrate assemblages

BOPA-FF was calculated from a total of 26,737 individuals belonging to the Capitellidae, Dorvilleidae, Glyceridae, Nereididae and Spionidae polychaete families (Martinez-Garcia *et al*., 2013); and from a total of 26,949 individuals belonging to 28 amphipod families (excluding the genus *Jassa* [Dauvin & Ruellet, 2007]); all of which were recorded from the NEF, SEF 1, and SEF 2 impacted and control plots during the study period.

The results of PERMANOVA indicated significant differences ($p < 0.0001$ except $p_{SEF\ 1} < 0.01$) in the BOPA-FF index for 'Im-vs-Co x Ti' at the NEF, SEF 1, and SEF 2 (Table 7.3). Mean values of the BOPA-FF index indicated 'Good' or 'High' EQS at the NEF, SEF 1 and SEF 2 impacted and control plots before tuna penning commenced. *A posteriori* comparisons indicated no significant differences in values of the BOPA-FF index between the impacted and control plots during this period (Figure 7.4; Appendix 2). The results of pair-wise tests showed that values of the BOPA-FF index recorded overall from the two NEF control plots before tuna penning commenced were significantly higher ($p < 0.001$) in March'01 compared with November'00. Following initiation of the tuna penning activities, values of the BOPA-FF index increased significantly at the NEF impacted plot in November'01 ($p < 0.001$), January'03 ($p < 0.05$), April'03 ($p_{vs\ November'00} < 0.01$, $p_{vs\ March'01} < 0.05$), November'03 ($p < 0.001$), March'04 ($p < 0.05$), and November'05 ($p_{vs\ November'00} < 0.001$, $p_{vs\ March'01} < 0.01$) (Figure 7.4; Appendix 2). The mean EQS at the NEF impacted plot was 'Bad' in November'01 and November'03, and 'Poor' in November'05; and pair-wise tests showed that BOPA-FF was significantly higher ($p < 0.001$) at the NEF impacted plot compared with the NEF control plots ('Good' EQS) in these periods. There were no significant differences in values of the BOPA-FF index between the mean 'Good' and 'Good' or 'High' EQS recorded respectively at the NEF impacted and control plots in

January'03, April'03, and March'04 following the tuna penning activities (Figure 7.4; Appendix 2). Values of BOPA-FF index recorded at the NEF impacted plot were significantly higher ($p < 0.001$ except $p_{\text{November'05 vs April'06}} < 0.05$ and $p_{\text{November'05 vs June'07}} < 0.01$) in November'01, November'03, and November'05, compared with subsequent sampling dates (April'06-April'09), and were significantly lower towards the end of the study period (May'08 & April'09) compared with the NEF control plots in the same periods ($p_{\text{May'08}} < 0.001$, $p_{\text{April'09}} < 0.05$), and with the NEF impacted plot towards the beginning the study period (November'01, January'03, April'03, & November'03) of ($p < 0.001$ except $p_{\text{January'03 vs May'08}} < 0.01$ & $p_{\text{January'03 vs April'09}} < 0.05$). No significant differences in values of the BOPA-FF index at the NEF impacted plot in May'08 and April'09, compared with November'00 and March'01 (before tuna penning commenced) were indicated by the statisical analysis (Figure 7.4; Appendix 2).

At the SEF 1, values of the BOPA-FF index increased significantly ($p < 0.01$ except $p_{\text{October'05}} < 0.001$) at the impacted plot in October'03, October'05, June'08, and June'09, following initiation of the tuna penning activities (Figure 7.4; Appendix 2). The mean EQS at the SEF 1 impacted plot was 'Moderate' in October'03 and June'09, 'Bad' in October'05, and 'Good' in June'08. *A posteriori* comparisons showed that values of the BOPA-FF index were significantly higher ($p < 0.001$ except $p_{\text{October'03}} < 0.01$) at the SEF 1 impacted plot compared with the SEF 1 control plots in October'03, October'05, and June'08, but no significant difference was indicated between the 'Moderate' and 'Good' mean EQS recorded respectively at the SEF 1 impacted and control plots in June'09 (Figure 7.4; Appendix 2). Values of the BOPA-FF index recorded at the SEF 1 impacted plot were significantly higher in October'05 compared with October'03 ($p < 0.01$), and in October'03, October'05 and June'08 compared with other sampling dates ($p < 0.001$ except $p_{\text{October'03 vs October'04}} < 0.01$ & $p_{\text{October'03 vs June'07; June'08 vs October'04 \& June'07}} < 0.05$) (Figure 7.4; Appendix 2).

At the SEF 2, values of the BOPA-FF index increased significantly ($p < 0.001$ except $p_{\text{Jun'02}} < 0.05$) in June'02, June'08 and June'09, and decreased significantly ($p < 0.001$ except $p_{\text{June'06}} < 0.05$) in June'03-June'06 at the impacted plot following initiation of

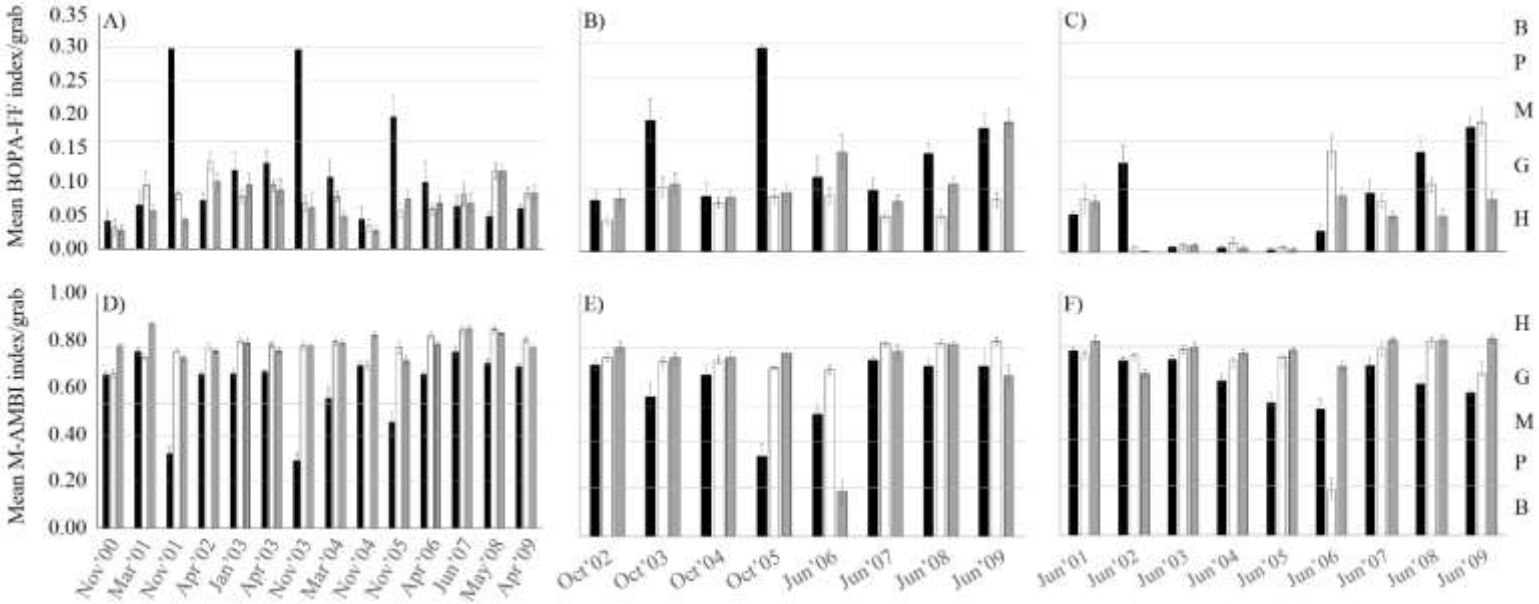


**Figure 7.4** Mean values (± SE) per grab for BOPA-FF and M-AMBI indices recorded from the impacted (black bars), 'Control 1' (white bars), and 'Control 2' (grey bars) plots of the (a, d) northeastern farm, (b, e) southeastern 'Farm 1', and (c, f) southeastern 'Farm 2'; showing the Ecological Quality Status (EQS) classification. H = 'High' EQS, G = 'Good' EQS, M = 'Moderate' EQS, P = 'Poor' EQS, and B = 'Bad' EQS

**Table 7.3** Results of three-factor, univariate, asymmetrical PERMANOVA for values of the BOPA-FF and M-AMBI indices, recorded from the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1), and southeastern 'Farm 2' (SEF 2). The F-ratio numerator/s and denominator/s are indicated. The level of significance was set at 0.05. Df = Degrees of freedom, RES = Residual, ns = not significant, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, F-Ratio Num = F-Ratio numerator, F-Ratio Den = F-Ratio denominator

| BOPA-FF | | | | | | |
|---|---|---|---|---|---|---|
| | NEF | | SEF 1 | | SEF 2 | |
| Source of Variation | df | p-value | df | p-value | df | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | ns | 1 | ns | 1 | ns |
| Control = Co | 1 | ns | 1 | * | 1 | ns |
| Time = Ti | 13 | ns | 7 | ns | 8 | ns |
| Im-vs-Co x Ti | 13 | **** | 7 | ** | 8 | **** |
| Co x Ti | 13 | ns | 7 | * | 8 | *** |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 42 | **** | 16 | ns | 27 | * |
| Site(Co x Ti) = Si(Co x Ti) | 84 | ns | 32 | ns | 54 | *** |
| RES: Si(Im-vs-Co x Ti) | 112 | | 48 | | 72 | |
| RES: Si(Co x Ti) | 224 | | 96 | | 144 | |
| Total | 503 | | 215 | | 323 | |
| M-AMBI | | | | | | |
| | NEF | | SEF 1 | | SEF 2 | |
| Source of Variation | df | p-value | df | p-value | df | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | *** | 1 | * | 1 | ** |
| Control = Co | 1 | ns | 1 | ns | 1 | ns |
| Time = Ti | 13 | ns | 7 | ns | 8 | ns |
| Im-vs-Co x Ti | 13 | **** | 7 | * | 8 | * |
| Co x Ti | 13 | **** | 7 | ns | 8 | * |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 42 | **** | 16 | ns | 27 | * |
| Site(Co x Ti) = Si(Co x Ti) | 84 | ns | 32 | ** | 54 | *** |
| RES: Si(Im-vs-Co x Ti) | 112 | | 48 | | 72 | |
| RES: Si(Co x Ti) | 224 | | 96 | | 144 | |
| Total | 503 | | 215 | | 323 | |

| Source of Variation | F-ratio Nom | F-ratio Den |
|---|---|---|
| Impact-vs-Control = Im-vs-Co | Im-vs-Co | Im-vs-Co x Ti |
| Control = Co | Co | Co x Ti |
| Time = Ti | Ti | Im-vs-Co x Ti + Co x Ti |
| Im-vs-Co x Ti | Im-vs-Co x Ti | Si(Im-vs-Co x Ti) |
| Co x Ti | Co x Ti | Si(Co x Ti) |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | Si(Im-vs-Co x Ti) | RES: Si(Im-vs-Co x Ti) |
| Site(Co x Ti) = Si(Co x Ti) | Si(Co x Ti) | RES: Si(Co x Ti) |
| RES: Si(Im-vs-Co x Ti) | | |
| RES: Si(Co x Ti) | | |

the tuna penning activities (Figure 7.4; Appendix 2). The mean EQS at the SEF 2 impacted plot was 'Good' in June'02, 'High' in June'03-June'06, 'Good' in June'08, and 'Moderate' in June'09; *a posteriori* comparisons showed that values of the BOPA-FF index were significantly higher ($p < 0.001$ except $p_{June'09} < 0.05$) in June'02, June'08 and June'09, but significantly lower ($p < 0.001$) in June'06, at the SEF 2 impacted plot compared with the SEF 2 control plots. No significant differences were indicated between the 'High' mean EQS's recorded at the SEF 2 impacted and control plots in June'03-June'05 (Figure 7.4; Appendix 2). Values of the BOPA-FF index recorded at the SEF 2 impacted plot in June'02, and in June'08 and June'09, were significantly higher ($p < 0.001$ except $p_{June'02,\ June'06} < 0.01$) compared with respectively June'03-June'06, and June'03, June'05 and June'06; while those recorded in June'06 were significantly lower ($p < 0.001$ except $p_{June'04,\ June'07} < 0.05$) compared with June'02, June'04 and June'07-June'09 (Figure 7.4; Appendix 2).

PERMANOVA indicated significant differences in values of the BOPA-FF index for 'Co x Ti' at the SEF 1 ($p < 0.05$) and SEF 2 ($p < 0.001$) (Table 7.3). It is worth noting that the variability in values of the BOPA-FF index among the southeastern control plots was high in June'09, when 'Good' and 'Moderate' mean EQS categorizations were recorded (Figure 7.4).

PERMANOVA also indicated significant differences in values of the BOPA-FF index for 'Si(Im-vs-Co x Ti)' at the NEF ($p < 0.0001$) and SEF 2 ($p < 0.05$), and for 'Si(Co x Ti)' at the SEF 2 ($p < 0.001$) (Table 7.3).

Values of the M-AMBI index were calculated using data from a total of 134,913 individuals, from 212 macroinvertebrate families, recorded from the NEF, SEF 1, and SEF 2 impacted and control plots during the study period. PERMANOVA indicated significant differences in values of the M-AMBI index for 'Im-vs-Co x Ti' at the NEF ($p < 0.0001$), SEF 1 ($p < 0.05$), and SEF 2 ($p < 0.05$) (Table 7.3).

*A posteriori* comparisons showed a significant difference ($p_{November'00} < 0.05$) in values of the M-AMBI index between the NEF impacted and control plots before tuna penning commenced, although the mean EQS at these plots was 'Good' in that period (Figure 7.4; Appendix 2). Values of the M-AMBI index recorded from the NEF

impacted ($p < 0.001$) and control ($p < 0.01$) plots were significantly lower in November'00 compared with March'01, before tuna penning commenced. Following initiation of the tuna penning activities, values of the M-AMBI index decreased significantly at the NEF impacted plot in November'01-April'09 ($p < 0.001$ except $p <$ $_{\text{March'01 vs January'03, November'04, April'09}}$ 0.01 & $p_{\text{March'01 vs June'07, May'08}} < 0.05$). Values of the M-AMBI index were significantly lower ($p < 0.001$ except $p_{\text{November'04}} < 0.05$) at the NEF impacted plot compared to the NEF control plots in November'01-April'09, while the mean EQS at the NEF impacted and control plots were respectively 'Poor' and 'Good' in November'01 and November'03, 'Moderate' and 'Good' in November'05, and 'Good' and 'High', or 'Good' and 'Good' during the rest of the study period (April'02-April'03, March'04, November'04, & April'06-April'09) (Figure 7.4; Appendix 2). Values of the M-AMBI index were significantly lower at the NEF impacted plot in November'01 compared with April'02-April'09 ($p < 0.001$ except $p_{\text{vs March'04}} < 0.01$ & $p_{\text{vs November'03, November '05}} < 0.05$), in November'03 compared with April'02, April'03, January'03, and March'04-April'09 ($p < 0.001$), and in November'05 compared with April'02, April'03, January'03, November'04 and April'06-April'09 ($p < 0.001$). No significant differences in values of the M-AMBI index were indicated at the NEF impacted plot towards the end of the study period (June'07-April'09) (Figure 7.4; Appendix 2).

At the SEF 1, values of the M-AMBI index were significantly lower ($p < 0.05$) at the impacted plot compared to the control plots before tuna penning commenced, although the mean EQS at these plots was 'Good' in that period (Figure 7.4; Appendix 2). Following initiation of the tuna penning activities, values of the M-AMBI index decreased significantly ($p < 0.001$ except $p_{\text{October'03}} < 0.05$) at the SEF 1 impacted plot in October'03, October'05, and June'06. Values of the M-AMBI index were significantly lower at the SEF 1 impacted plot compared to the SEF 1 control plots in October'03 ($p < 0.01$) and October'05 ($p < 0.001$), while the mean EQS was respectively 'Good' and 'Good', and 'Poor' and 'Good' in these periods. Values of the M-AMBI index were significantly higher at the SEF 1 impacted plot compared to the SEF 1 control plots in June'06 ($p < 0.001$), while the mean EQS was 'Moderate' at both plots in this period. No significant differences in values of the M-AMBI index were indicated at the SEF 1 impacted plot in October'04 and June'07-June'09, compared with October'02 (before tuna penning commenced) (Figure 7.4; Appendix

2). Values of the M-AMBI index were significantly lower at the SEF 1 impacted plot in: (i) October'03 compared with June'07 ($p < 0.05$); (ii) October'05 compared with October'03 and June'06-June'09 ($p < 0.001$ except $p_{\text{vs June'09}} < 0.01$ & $p_{\text{vs June'06}} < 0.05$); and (iii) June'06 compared with subsequent sampling dates (June'07-June'09) ($p < 0.001$ except $p_{\text{vs June'09}} < 0.05$). There were no significant differences in values of the M-AMBI index at the SEF 1 impacted plot towards the end of the study period (June'07-June'09) (Figure 7.4; Appendix 2).

At the SEF 2, no significant difference was indicated in values of the M-AMBI index between impacted and control plots before tuna penning commenced, and the mean EQS at each of these plots was 'Good' in this period (Figure 7.4; Appendix 2). Following initiation of the tuna penning activities, values of the M-AMBI index decreased significantly ($p < 0.001$ except $p_{\text{June'04}} < 0.01$) at the SEF 2 impacted plot in June'04-June'06, June'08, and June'09, and were significantly lower ($p < 0.001$) at the SEF 2 impacted plot compared to the SEF 2 control plots in the same periods (Appendix 2). The mean EQS was 'Good' at both SEF 2 impacted and control plots in the same periods, with the exception of June'06, when 'Moderate' and 'Good' EQS were recorded (Figure 7.4). Pair-wise tests showed that values of the M-AMBI index were significantly lower ($p < 0.001$ except $p_{\text{June'04}} < 0.05$ & $p_{\text{June'07}} < 0.01$) at the SEF 2 impacted plot in June'06 compared with June'02, June'03, June'04, and June'07 (Figure 7.4; Appendix 2).

PERMANOVA indicated significant differences in values of the M-AMBI index for 'Co x Ti' at the NEF ($p < 0.0001$) and SEF 2 ($0 < 0.05$) (Table 7.3), while the variability in values of the index among the SEF's control plots was high in June'06, when 'Good' and 'Bad' mean EQS categorizations were recorded (Figure 7.4).

PERMANOVA also indicated significant differences in values of the M-AMBI index for 'Si(Im-vs-Co x Ti)' at the NEF ($p < 0.0001$) and SEF 2 ($0 < 0.05$), and for 'Si(Co x Ti)' at the SEF 1 ($p < 0.01$) and SEF 2 ($p < 0.001$) (Table 7.3).

### 7.3.2 Multivariate data analyses

#### 7.3.2(i) Sediment physico-chemical attributes

The results of PCO ordination explained 56.5% of the total variation in sediment physico-chemical data recorded from the NEF, and indicated separation between samples collected from the impacted plot in November'01, and all the other samples collected from the NEF impacted and control plots during the study period (Figure 7.5). PCO ordinations for SEF 1 and SEF 2 explained respectively 65.0% and 49.9% of the total variation in sediment physico-chemical data, and indicated separation between samples collected from the impacted and control plots in June'09, and samples collected from the impacted and control plots during the rest of the study period (Figure 7.5). High dispersion of samples characterized the southeastern farms' impacted and control plots in June'09. PCO ordination of sediment physico-chemical data recorded from SEF 1 also indicated separation between: (i) a group of samples collected from the impacted plot before tuna penning commenced (October'02) and from the control plots in October'02-June'08, and (ii) a group of samples collected from the impacted plot after the tuna penning activities (October'03-June'09). PCO ordination of sediment physico-chemical data from SEF 2 also indicated separation between: (i) a group of samples collected from the 'Control 2' plot in June'02-June'04, and (ii) a group of samples collected from the impacted and 'Control 1' plots in June'01-June'08 and from the 'Control 2' plot in June'01 and June'05-June'08 (Figure 7.5).

Multivariate PERMANOVA showed that the square root estimates of 'Im-vs-Co x Ti' (0.89799 $_{NEF}$, 0.347208 $_{SEF\ 1}$, & -0.1542 $_{SEF\ 2}$) and 'Co x Ti' (-0.22578 $_{NEF}$, 0.075532 $_{SEF\ 1}$, & 0.36328 $_{SEF\ 2}$) as components of variation in sediment physico- chemical data recorded from the NEF, SEF 1, and SEF 2, were negative or small (Table 7.4). PERMDISP indicated significant differences in sediment physico-chemical data for 'Im-vs-Co x Ti' ($p < 0.001$ except $p_{SEF\ 2} < 0.01$) at the NEF, SEF 1 and SEF 2, while PERMANOVA did not (Table 7.4).

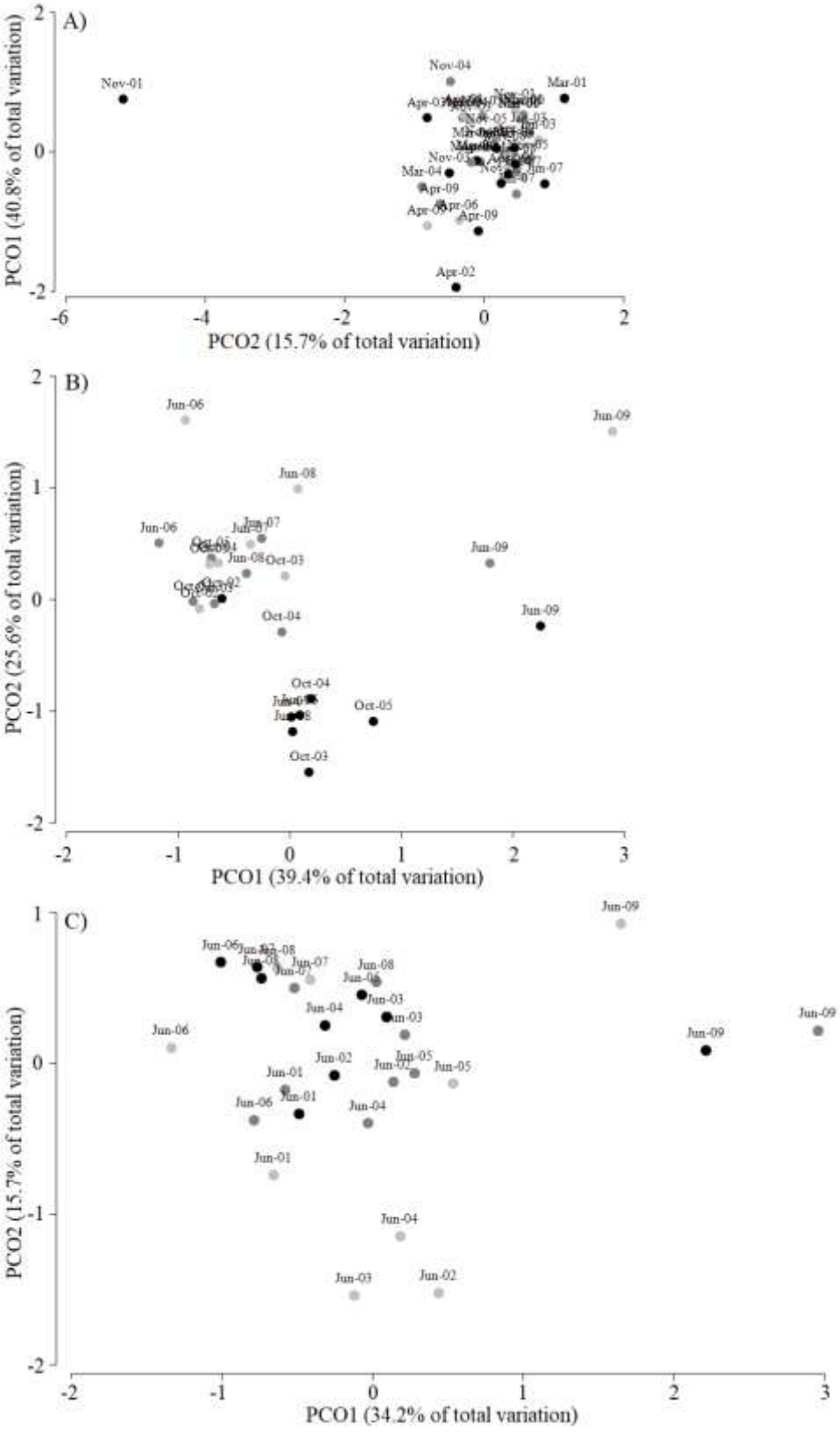


**Figure 7.5** PCO plots for sediment physico-chemical attributes derived from normalised environmental data collected from (a) the northeastern farm, (b) southeastern 'Farm 1', and (c) southeastern 'Farm 2' at impacted (black), 'Control 1' (dark grey), and 'Control 2' (pale grey) plots during the study period.

**Table 7.4** Results of two-factor, multivariate, asymmetrical PERMANOVA and PERMDISP tests for sediment phyisco-chemical data. Variables included in the analyses are normalised values of mean sediment grain size (phi), and percent organic carbon and organic nitrogen content in the sediment. The level of significance was set at 0.05. Df = Degrees of freedom, Sq Rt Var = Square Root Estimate of Component of Variation, RES = Residual, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | | NEF | | |
|---|---|---|---|---|
| | | | PERMANOVA | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | 0.17194 | ns | *** |
| Control = Co | 1 | 0.2085 | ns | ns |
| Time = Ti | 13 | 0.7382 | ns | ** |
| Im-vs-Co x Ti | 13 | 0.89799 | ns | *** |
| Co x Ti | 13 | -0.2258 | ns | ** |
| RES: Im-vs-Co x Ti | 42 | 0.4095 | | |
| RES: Co x Ti | 84 | 1.0439 | | |
| Total | 167 | 3.24425 | | |
| | | SEF 1 | | |
| | | | PERMANOVA | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | 0.53045 | * | ns |
| Control = Co | 1 | 0.3187 | ns | ns |
| Time = Ti | 7 | 1.0041 | ns | ns |
| Im-vs-Co x Ti | 7 | 0.347208 | ns | *** |
| Co x Ti | 7 | 0.075532 | ns | * |
| RES: Im-vs-Co x Ti | 17 | 0.28314 | | |
| RES: Co x Ti | 32 | 0.95616 | | |
| Total | 72 | 3.51529 | | |
| | | SEF 2 | | |
| | | | PERMANOVA | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | 0.52238 | * | ns |
| Control = Co | 1 | -0.0586 | ns | ns |
| Time = Ti | 8 | 1.048 | * | ** |
| Im-vs-Co x Ti | 8 | -0.1542 | ns | ** |
| Co x Ti | 8 | 0.36328 | ns | ** |
| RES: Im-vs-Co x Ti | 27 | -0.0437 | | |
| RES: Co x Ti | 54 | 1.4015 | | |
| Total | 107 | 3.07869 | | |

*A posteriori* comparisons indicated significant differences ($p < 0.01$) in dispersion of samples of sediment physico-chemical data between impacted and control plots at the NEF and SEF 1 before tuna penning commenced (Appendix 3). No significant difference in the dispersion of samples of sediment physico-chemical data was

indicated between the SEF 2 impacted and control plots in the same period. Following initiation of the tuna penning activities, the dispersion of samples of sediment physico-chemical data differed significantly ($p < 0.05$) at the NEF impacted plot in November'01, April'03, March'04-April'06, and April'09 compared with that recorded before tuna penning commenced, and with that recorded at the NEF control plots in November'01 ($p < 0.01$) and April'03 ($p < 0.05$). There were no significant differences in the dispersion of samples of sediment physico-chemical data between the NEF impacted and control plots in March'04-April'06 and April'09. The dispersion of samples of sediment physico-chemical data at the NEF impacted plot differed significantly in November'01 compared with April'02, January'03, November'03, and June'07-April'09 ($p < 0.05$), and in April'03 compared with May'08 ($p < 0.05$) (Appendix 3).

At the SEF 1, dispersion of samples of sediment physico-chemical data differed significantly ($p < 0.05$) at the impacted plot in October'04-June'08 compared with October'02 (before tuna penning commenced), but no significant differences were detected between the impacted and control plots in that period (October'04-June'08) (Appendix 3). At the SEF 2, no significant difference in the dispersion of samples of sediment physico-chemical data was indicated at the impacted plot during the study period (June'02-June'09) compared with that recorded at this plot before tuna penning commenced, and at the control plots in the same periods (Appendix 3).

PERMDISP also indicated significant differences in sediment physico-chemical data for 'Co x Ti' ($p < 0.01$ except $p_{SEF\ 1} < 0.05$) at the NEF, SEF 1, and SEF 2, while PERMANOVA did not (Table 7.4).

### 7.3.2(ii) Macroinvertebrate assemblages

PCO ordination explained 33.3% and 29.6% of the total variation in polychaete and amphipod family abundance recorded from the NEF, and indicated high dissimilarity between November'01 impacted samples, November'03 impacted samples, November'05 and March'04 impacted samples, and all other NEF samples collected during the study (Figure 7.6). Polychaete family abundance also showed separation (in decreasing order of dissimilarity) at the NEF between: (i) a group of April'02 and

January'03 impacted samples; (ii) a group of April'06-April'09 impacted samples, and control samples collected after the tuna penning activities (November'01-March'04 & November'05-April'09); and (iii) a group of impacted and control samples collected before the tuna penning (November'00 & March'01) and afterward in November'04 (Figure 7.6). Amphipod family abundance also showed separation (in decreasing order of dissimilarity) at the NEF between: (i) May'08 impacted samples; (ii) a group of impacted samples collected in April'02-November'03, April'06, January'07 and April'09; (iii) a group of impacted and control samples collected in spring before the tuna penning (March'01) and control samples collected afterward (November'01-March'04 & November'05-April'09); and (iv) a group of impacted and control samples collected in autumn before the tuna penning (November'00) and afterward in November'04 (Figure 7.6).

For the SEF 1, PCO ordination explained 28.4% of the total variation in polychaete family abundance, and indicated separation (in decreasing order of dissimilarity) between: (i) June'06 impacted samples; (ii) a group of June'06 and June'09 'Control 2' samples; (iii) a group of October'02-October'05 and June'07-June'09 impacted samples; and (iv) the remaining control samples collected during the study period (Figure 7.6). PCO ordination explained 30.9% of the total variation in amphipod family abundance recorded from the SEF 1, and indicated separation (in decreasing order of dissimilarity) between the: (i) October'05 impacted samples; (ii) June'06 impacted samples; (iii) October'03 impacted samples; and (iv) a group of October'04 and June'07-June'09 impacted samples, October'03 and October'05-June'09 'Control 1' samples, and October'03-June'09 'Control 2' samples, and impacted and control samples collected before tuna penning commenced (October'02) (Figure 7.6).

For the SEF 2 data, PCO ordination explained 21.1% of the total variation in polychaete family abundance, and indicated separation (in decreasing order of dissimilarity) between: (i) June'06 impacted samples; (ii) a group of June'07-June'09 impacted and 'Control 1' samples; (iii) June'02 'Control 2' samples; (iv) June'06 'Control 2' samples; and (vi) a group of impacted and control samples collected before tuna penning commenced (June'01), and afterwards from the impacted plot in June'02-June'04, from the 'Control 1' plot in June'02-June'05, and from the 'Control 2' plot

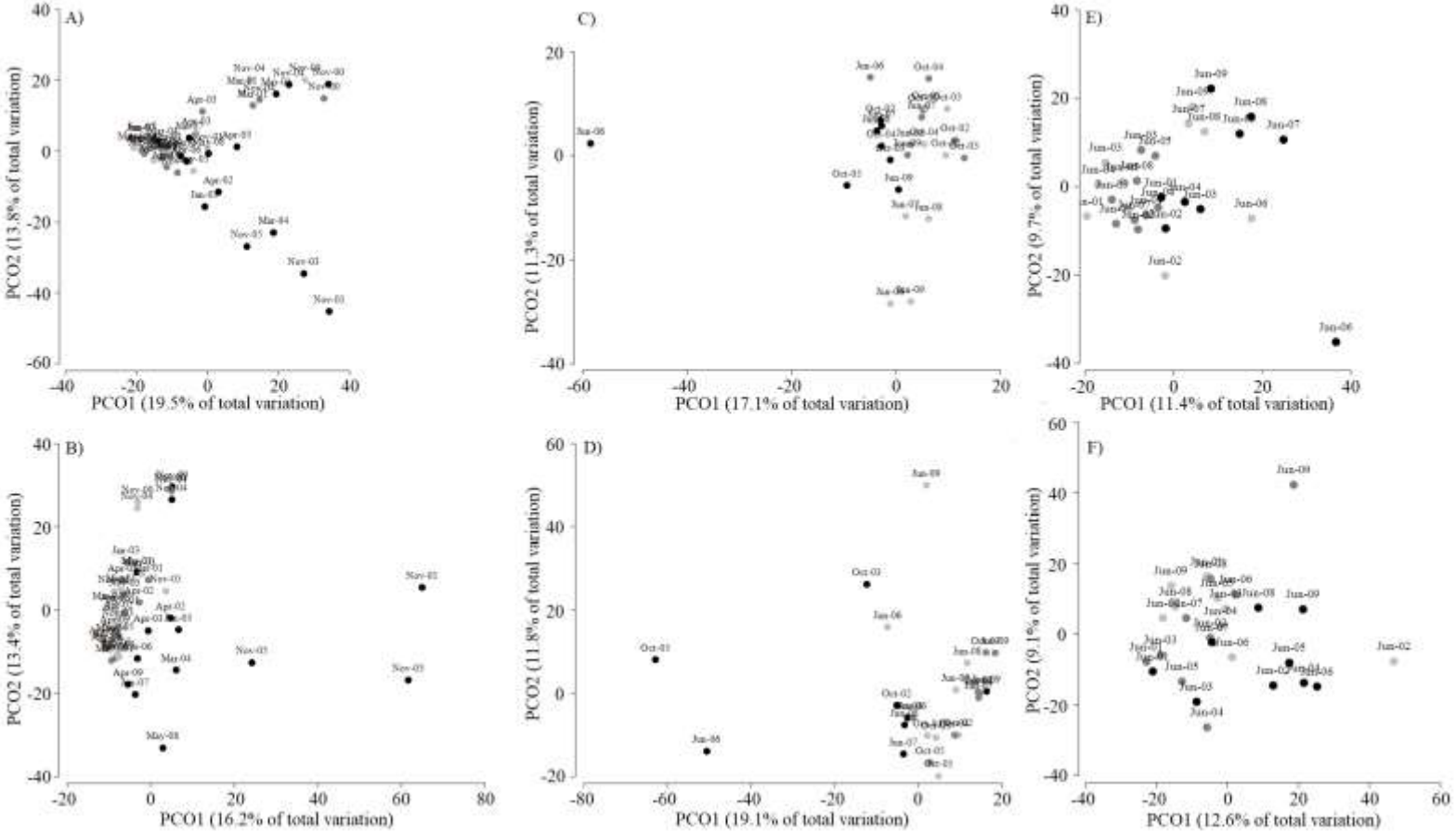


**Figure 7.6** PCO plots of fourth-root transformed (a, c, e) polychaete and (b, d, f) amphipod family abundance data collected from the (a, b) northeastern farm, (c, d) southeastern 'Farm 1', and (e, f) southeastern 'Farm 2' impacted (black), 'Control 1' (dark grey), and 'Control 2' (light grey) plots during the study.

throughout the study (June'02-June'09) (Figure 7.6). PCO ordination explained 21.7% of the total variation in amphipod family abundance recorded from the SEF 2, and indicated separation (in decreasing order of dissimilarity) between: (i) June'09 'Control 1' samples; (ii) June'02 'Control 2' samples; (iii) a group of June'02 and June'04-June'06 impacted samples; (iv) June'09 impacted samples; (v) June'08 impacted samples; (vi) a group of June'07 impacted samples, June'03-June'09 'Control 1' samples, and June'02 and June'06-June'08 'Control 2' samples; and (v) a group of samples collected from the impacted and control plots before tuna penning commenced (June'01), and afterward from the impacted and 'Control 2' plot respectively in June'03 and June'03-June'05 (Figure 7.6).

Multivariate PERMANOVA showed that the square root estimates of the terms 'Im-vs-Co x Ti' and 'Co x Ti' as components of variation in polychaete (respectively 8.0319 & 6.9111 $_{NEF}$, 0.996 & 11.303 $_{SEF\ 1}$, and -2.29 & 14.388 $_{SEF\ 2}$) and amphipod (respectively 5.7154 & 7.4656 $_{NEF}$, 3.922 & 11.27 $_{SEF\ 1}$, and -3.476 & 18.313 $_{SEF\ 2}$) family abundance, were small compared to that of the residual variation ('Si(Co x Ti)') (respectively 23.1 & 27.326 $_{NEF}$, 25.48 & 33.123 $_{SEF\ 1}$, and 86.047 & 98.783 $_{SEF\ 2}$ for polychaetes and amphipods) (Table 7.5). Both PERMANOVA and PERMDISP tests indicated significant differences ($p < 0.001$ except $p_{PERMANOVA\ Amphipods} < 0.05$) in polychaete and amphipod family abundance for 'Im-vs-Co x Ti' at the NEF. At the southeastern farms, PERMDISP indicated significant differences ($p < 0.001$ except $p_{SEF\ 1\ Polychaetes} < 0.01$) in polychaete and amphipod family abundance for 'Im-vs-Co x Ti', while PERMANOVA did not (Table 7.5).

*A posteriori* comparisons of PERMANOVA and PERMDISP tests showed no significant differences in polychaete and amphipod family abundance between the NEF impacted and control plots before tuna penning commenced (November'00 & March'01), with the exception of polychaete family abundance in March'01 ($p_{PERMDISP} < 0.01$) (Appendix 4). Polychaete and amphipod family abundance differed significantly ($p < 0.001$ except $p_{Polychaetes\ PERMDISP\ Im} < 0.05$ & $p_{Amphipods\ PERMANOVA\ Im} < 0.01$) between November'00 and March'01 at the NEF impacted and control plots before tuna penning commenced. Following initiation of the tuna penning activities, polychaete and amphipod family abundance differed significantly at the NEF impacted

**Table 7.5** Results of three-factor, multivariate, asymmetrical PERMANOVA and PERMDISP tests for polychaete and amphipod assemblages. Variables included in the analysis are fourth-root transformed family abundance values. Level of significance set at 0.05. Df = Degrees of freedom, Sq Rt Var = Square Root Estimate of Component of Variation, RES = Residual, NEF = northeastern farm, SEF 1 = southeastern 'Farm 1', SEF 2 = southeastern 'Farm 2', ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | | Polychaetes | | | Amphipods | | |
|---|---|---|---|---|---|---|---|
| | | NEF | | | | | |
| | | PERMANOVA | | PERMDISP | PERMANOVA | | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value | Sq Rt Var | p-value | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | 8.1583 | * | *** | 8.2087 | * | *** |
| Control = Co | 1 | 4.5357 | ns | ns | 3.2703 | ns | ns |
| Time = Ti | 13 | 21.273 | * | *** | 17.751 | ns | *** |
| Im-vs-Co x Ti | 13 | 8.0319 | *** | *** | 5.7154 | * | *** |
| Co x Ti | 13 | 6.9111 | ns | *** | 7.4656 | ns | *** |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 42 | 2.3155 | * | *** | 3.6737 | ns | *** |
| Site(Co x Ti) = Si(Co x Ti) | 84 | 8.2335 | * | *** | 6.0725 | ns | *** |
| RES: Si(Im-vs-Co x Ti) | 112 | 2.399 | | | 5.533 | | |
| RES: Si(Co x Ti) | 224 | 23.1 | | | 27.326 | | |
| Total | 503 | 84.958 | | | 85.0162 | | |

**Table 7.5** Continued

| | | Polychaetes | | | Amphipods | | |
|---|---|---|---|---|---|---|---|
| | | SEF 1 | | | | | |
| | | PERMANOVA | | PERMDISP | PERMANOVA | | PERMDISP |
| Source of Variation | df | Sq Rt Var | p-value | p-value | Sq Rt Var | p-value | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | 3.0774 | ns | *** | 4.9313 | ns | *** |
| Control = Co | 1 | 8.6876 | ns | ns | 7.5387 | ns | *** |
| Time = Ti | 7 | 11.222 | ns | *** | 15.599 | ns | *** |
| Im-vs-Co x Ti | 7 | 0.996 | ns | *** | 3.922 | ns | *** |
| Co x Ti | 7 | 11.303 | ns | ** | 11.27 | ns | *** |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 16 | 2.8327 | * | *** | 1.8 | ns | ** |
| Site(Co x Ti) = Si(Co x Ti) | 32 | 9.8113 | ns | ** | 15.631 | * | ** |
| RES: Si(Im-vs-Co x Ti) | 48 | 0.942 | | | 3.283 | | |
| RES: Si(Co x Ti) | 96 | 25.48 | | | 33.123 | | |
| Total | 215 | 74.352 | | | 97.098 | | |
| | | SEF 2 | | | | | |
| | | PERMA | | PERMD | PERMA | | PERMD |
| Source of Variation | df | Sq Rt Var | p-value | p-value | Sq Rt Var | p-value | p-value |
| Impact-vs-Control = Im-vs-Co | 1 | 5.022 | * | *** | 1.3096 | ns | ns |
| Control = Co | 1 | 5.052 | ns | ns | 9.5224 | ns | ns |
| Time = Ti | 8 | 12.415 | ns | *** | 13.651 | ns | *** |
| Im-vs-Co x Ti | 8 | -2.29 | ns | *** | -3.476 | ns | *** |
| Co x Ti | 8 | 14.388 | * | *** | 18.313 | * | *** |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 27 | 3.631 | *** | *** | 0.216 | * | ** |
| Site(Co x Ti) = Si(Co x Ti) | 54 | 16.343 | ** | *** | 19.844 | ** | *** |
| RES: Si(Im-vs-Co x Ti) | 72 | 1.09 | | | 1.204 | | |
| RES: Si(Co x Ti) | 144 | 30.396 | | | 38.199 | | |
| Total | 323 | 86.047 | | | 98.783 | | |

plot in November'01-April'09 compared with November'00 and March'01 (see Appendix 4) (with the exception of amphipod family abundance in November'04 [$p_{\text{November'00 vs November'04}} > 0.05$]), and at the NEF impacted plot compared with the NEF control plots in November'01-March'04 and November'05-April'09 ($p < 0.001$ except $p_{\text{Amphiphods PERMDISP April'03}} < 0.05$ & $p_{\text{Amphiphods PERMDISP April'06}} < 0.01$) (Appendix 4). Polychaete and amphipod family abundance recorded at the NEF impacted plot after initiation of the tuna penning activities differed significantly in each sampling date compared with the subsequent sampling dates (e.g. in November'01 compared with April'02-April'09; in April'02 compared with January'03-April'09…in May'08 compared with April'09) (see Appendix 4).

At the SEF 1, the dispersion of samples of polychaete family abundance differed significantly ($p < 0.01$) between the impacted and control plots before tuna penning commenced (October'02), while that of samples of amphipod family abundance did not differ significantly in the same period (Appendix 4). Following initiation of the tuna penning activities, the dispersion of samples of polychaete family abundance data differed significantly at the SEF 1 impacted plot in October'03 ($p < 0.05$), June'06 ($p < 0.001$), and June'08 ($p < 0.01$), compared with October'02, but no significant differences were detected between the SEF 1 impacted and control plots in the same period (Appendix 4). The dispersion of samples of amphipod family abundance at the SEF 1 impacted plot following initiation of the tuna penning activities in October'03 and October'05 differed significantly ($p < 0.01$ except $p_{\text{October'05 Im-vs-Co}} < 0.001$ & $p_{\text{October'03 \& October '05 vs June'09}} < 0.05$) compared with: (i) October'02; (ii) the SEF 1 control plots in the same periods; and (iii) the SEF 1 impacted plot in June'07-June'09 (Appendix 4).

At the SEF 2, dispersion of samples of polychaete and amphipod family abundance differed significantly ($p < 0.05$) between the impacted and control plots before tuna penning commenced (June'01) (Appendix 4). Following initiation of the tuna penning activities, dispersion of samples of polychaete family abundance differed significantly at the SEF 2 impacted plot in June'02-June'08 compared with June'01 ($p < 0.001$ except $p_{\text{vs June'02}} < 0.05$ & $p_{\text{vs June'03, June'08}} < 0.01$), and at the SEF 2 impacted plot compared with the SEF 2 control plots in June'02, June'03, and June'05-June'08 ($p < 0.05$ except $p_{\text{June'06, June '07}} < 0.01$ & $p_{\text{June'08}} < 0.001$). No significant differences in the

dispersion of samples of polychaete family abundance were detected between the SEF 2 impacted and control plots in June'04 and June'09 (Appendix 4). The dispersion of samples of polychaete family abundance at the SEF 2 impacted plot differed significantly in: June'02 compared with June'04-June'06 ($p < 0.05$ except $p_{June'06} < 0.001$); June'06 compared with June'03 ($p < 0.01$), June'05 ($p < 0.05$), June'07 ($p < 0.05$) and June'09 ($p < 0.0001$); and June'09 compared with June'03-June'08 ($p_{vs\ June'03,\ June'08} < 0.05$, $p_{vs\ June'04,\ June\ '07} < 0.01$ & $_{vs\ June'05,\ June'06} < 0.001$) (Appendix 4). The dispersion of samples of amphipod family abundance differed significantly at the SEF 2 impacted plot in June'02 ($p < 0.05$), June'04 ($p < 0.0001$), June'05 ($p < 0.01$), June'08 ($p < 0.01$), and June'09 ($p < 0.001$), compared with June'01, and at the SEF 2 impacted plot compared with the SEF 2 control plots in June'04 ($p < 0.01$) and June'08 ($p < 0.0001$). No significant differences in the dispersion of samples of amphipod family abundance were detected between the SEF 2 impacted and control plots in June'02, June'05, and June'09 (Appendix 4). The dispersion of samples of amphipod family abundance differed significantly ($p < 0.05$ except $p_{June'04,\ June\ '03} < 0.0001$) at the SEF 2 impacted plot in June'04 and June'08 compared with respectively June'02, June'03 and June'05; and with June'03 and June'04 (Appendix 4).

PERMDISP indicated a significant difference in polychaete and amphipod family abundance for 'Co x Ti' ($p < 0.001$ except $p_{SEF\ 1\ Polychaetes} < 0.01$) at the NEF and SEF 1, while PERMANOVA did not. On the other hand, both PERMANOVA ($p < 0.05$) and PERMDISP ($p < 0.001$) tests indicated a significant difference in polychaete and amphipod family abundance for 'Co x Ti' at the SEF 2 (Table 7.5). Significant differences were also detected for 'Si(Im-vs-Co x Ti)' ($p < 0.001$ except $p_{PERMANOVA\ Polychaetes\ NEF\ \&\ SEF\ 1,\ Amphipods\ SEF\ 2} < 0.05$) and 'Si(Co x Ti)' ($p < 0.001$ except $p_{PERMANOVA\ Polychaetes\ NEF,\ Amphipods\ SEF\ 1} < 0.05$ & $p_{Polychaetes,\ Amphipods\ PERMDISP\ SEF\ 1,\ PERMANOVA\ SEF\ 2} < 0.01$) in polychaete and amphipod family abundance at each of the investigated tuna farms (Table 7.5).

SIMPER analysis of family abundance collected from the NEF showed high dissimilarity between impacted and control plots in November'01 (c. 73% $_{Polychaetes}$ and c. 92% $_{Amphipods}$), November'03 (c. 65% $_{Polychaetes}$ and c. 90% $_{Amphipods}$) and November'05 (c. 58% $_{Polychaetes}$ and c. 72% $_{Amphipods}$), particularly in the case of amphipod assemblages (Table 7.6). The top three polychaete families contributing

most to this dissimilarity (in terms of number of individuals) were: Capitellidae and Dorvilleidae, which were more abundant at the impacted plot compared to the control plots; and Paraonidae, Hesionidae, and Maldanidae; which were less abundant at the impacted plot compared to the control plots. It is noteworthy that capitellid polychaetes contributed to around 20 % of this dissimilarity in November'01 and November'03; while capitellids contributed around 11 %, and dorvilleids contributed around 7 % of this dissimilarity in November'05. The top three amphipod families contributing most to this dissimilarity (i.e. Urothoidae, Phoxocephalidae, and Lysianassidae) were less abundant at the impacted plot compared with the control plots. The dissimilarity in polychaete (c. 35 – 49 %) and amphipod (c. 36 – 56 %) family abundance between the NEF impacted and control plots was low during the rest of the study period (November'00, March'01, April'02, January'03, November'04, & April'06-April'09) (Table 7.6).

At the SEF 1, SIMPER analysis of polychaete family abundance showed high dissimilarity (c. 71%) between the impacted and control plots in June'06 (Table 7.6). The polychaetes that contributed most to this dissimilarity (i.e. Glyceridae, Maldanidae, and Paraonidae) were less abundant at the impacted plot compared to the control plots. The dissimilarity in polychaete family abundance between the SEF 1 impacted and control plots in October'02-October'05 and June'07-June'09, was low (c. 39 – 49 %), but capitellid polychaetes contributed around 18 % of this dissimilarity in October'05 (Table 7.6). For amphipod family abundance, SIMPER analysis showed high dissimilarity between the SEF 1 impacted and control plots in October'03 (c. 69 %), October'05 (c. 84 %), June'06 (c. 78 %), and June'09 (c. 65 %) (Table 7.6). The amphipods that contributed most to this dissimilarity in October'03, October'05, and June'06 (i.e. Lysianassidae, Ampeliscidae, and Photidae or Urothoidae) were, in general, less abundant at the impacted plot compared to the control plots; while those that contributed most to this dissimilarity in June'09 (i.e. Photidae, Caprellidae, and Lysianassidae) were less abundant at the control plots compared to the impacted plot. The dissimilarity in amphipod family abundance between the SEF 1 impacted and control plots was low (c. 52 – 55 %) during the rest of the study period (October'02, October'04, & October'08) (Table 7.6).

**Table 7.6** Results of SIMPER analysis showing the top three families (in terms of abundance) contributing to the dissimilarity of samples recorded for polychaete and amphipod assemblages between impacted and control plots at the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1), and southeastern 'Farm 2' (SEF 2) during the study period. Avg Diss (%) = Average Dissimilarity (%), Avg Abund = Average Abundance, Contrib (%) = Contribution (%)

| NEF | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | | | | Amphipods | | | | |
| | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) |
| November'00 | 50.17 | Terebellidae | **0.91** | 0.66 | 8.20 | 48.50 | Maeridae | 0.51 | **0.63** | 12.54 |
| | | Capitellidae | 0.79 | **0.83** | 7.99 | | Urothoidae | 1.09 | **1.12** | 12.42 |
| | | Lumbrineridae | **0.77** | 0.74 | 7.85 | | Lysianassidae | 0.91 | **1.08** | 11.10 |
| March'01 | 35.80 | Nereididae | 0.94 | **1.27** | 8.74 | 42.07 | Urothoidae | 1.10 | **1.16** | 11.43 |
| | | Phyllodocidae | 0.40 | **0.88** | 8.71 | | Ischyroceridae | 0.33 | **0.76** | 11.07 |
| | | Lumbrineridae | **0.99** | 0.72 | 8.02 | | Ampeliscidae | 0.82 | **1.18** | 9.16 |
| November'01 | 73.10 | Capitellidae | **3.54** | 1.04 | 21.01 | 91.97 | Urothoidae | 0.00 | **1.47** | 18.57 |
| | | Paraonidae | 0.49 | **1.77** | 10.82 | | Phoxocephalidae | 0.17 | **1.47** | 16.79 |
| | | Hesionidae | 0.00 | **1.1** | 9.25 | | Lysianassidae | 0.17 | **1.22** | 14.71 |
| April'02 | 48.01 | Hesionidae | 0.00 | **1.24** | 11.40 | 42.75 | Phoxocephalidae | 0.54 | **1.23** | 15.57 |
| | | Capitellidae | 0.52 | **1.37** | 8.43 | | Ampeliscidae | 0.46 | **1.02** | 14.72 |
| | | Maldanidae | 0.25 | **1.03** | 7.91 | | Oedicerotidae | 0.87 | **1.12** | 10.77 |
| January'03 | 45.12 | Dorvilleidae | **1.86** | 0.30 | 11.84 | 55.72 | Lysianassidae | **2.19** | 1.12 | 18.85 |
| | | Hesionidae | 0.00 | **1.12** | 8.36 | | Phoxocephalidae | 0.45 | **1.34** | 14.66 |
| | | Sabellidae | 0.43 | **1.37** | 7.43 | | Urothoidae | 0.90 | **1.29** | 10.99 |
| April'03 | 41.95 | Orbiniidae | **1.92** | 0.59 | 7.96 | 50.18 | Phoxocephalidae | 0.68 | **1.67** | 10.55 |
| | | Maldanidae | 0.17 | **1.41** | 7.91 | | Urothoidae | 0.76 | **1.18** | 9.19 |
| | | Cirratulidae | **2.53** | 1.28 | 7.52 | | Photidae | 0.62 | **0.98** | 7.67 |

**Table 7.6** Continued

| NEF | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Polychaetes | | | | | | Amphipods | | | | |
| | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) |
| November'03 | 64.52 | Capitellidae | **3.69** | 0.82 | 19.68 | 89.65 | Phoxocephalidae | 0.00 | **1.50** | 17.86 |
| | | Maldanidae | 0.08 | **1.40** | 9.23 | | Lysianassidae | 0.18 | **1.40** | 14.87 |
| | | Hesionidae | 0.00 | **1.20** | 8.32 | | Urothoidae | 0.08 | **1.30** | 14.60 |
| March'04 | 57.64 | Hesionidae | 0.00 | **1.50** | 11.05 | 52.79 | Phoxocephalidae | 0.41 | **1.50** | 15.69 |
| | | Sabellidae | 0.17 | **1.50** | 10.07 | | Urothoidae | 0.65 | **1.30** | 12.91 |
| | | Maldanidae | 0.17 | **1.50** | 9.60 | | Photidae | 0.38 | **1.20** | 12.08 |
| November'04 | 48.28 | Hesionidae | 0.49 | **1.00** | 9.31 | 44.00 | Urothoidae | 1.09 | **1.10** | 14.85 |
| | | Sabellidae | 0.79 | **1.00** | 8.83 | | Ampeliscidae | 0.58 | **0.90** | 13.15 |
| | | Eunicidae | **0.96** | 0.71 | 8.46 | | Phoxocephalidae | 1.16 | **1.50** | 11.36 |
| November'05 | 57.81 | Capitellidae | **1.82** | 1.01 | 11.44 | 72.42 | Phoxocephalidae | 0.10 | **1.20** | 14.67 |
| | | Maldanidae | 0.31 | **1.30** | 9.08 | | Lysianassidae | 0.51 | **1.20** | 13.27 |
| | | Dorvilleidae | **1.10** | 0.40 | 7.21 | | Urothoidae | 0.69 | **1.20** | 12.64 |
| April'06 | 49.12 | Hesionidae | 0.10 | **1.50** | 10.12 | 42.35 | Photidae | 0.66 | **1.90** | 16.41 |
| | | Capitellidae | 0.48 | **1.00** | 5.90 | | Phoxocephalidae | 0.71 | **1.60** | 12.55 |
| | | Dorvilleidae | **1.00** | 0.30 | 5.83 | | Urothoidae | 1.07 | **1.60** | 11.69 |
| June'07 | 39.93 | Hesionidae | 0.00 | **1.40** | 10.07 | 47.56 | Phoxocephalidae | 0.27 | **1.40** | 11.22 |
| | | Aphroditidae | 0.31 | **1.20** | 6.87 | | Photidae | 1.04 | **2.10** | 10.61 |
| | | Nereididae | 0.76 | **1.70** | 6.82 | | Aoridae | 0.32 | **1.00** | 8.29 |
| May'08 | 48.37 | Maldanidae | 0.47 | **1.80** | 9.00 | 49.12 | Phoxocephalidae | 0.17 | **1.40** | 13.44 |
| | | Hesionidae | 0.00 | **1.30** | 8.50 | | Urothoidae | 0.11 | **1.10** | 11.06 |
| | | Nereididae | 0.71 | **1.80** | 7.46 | | Stenothoidae | **0.80** | 0.00 | 8.97 |

**Table 7.6** Continued

| | NEF | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | | | | Amphipods | | | | |
| | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) |
| April'09 | 35.44 | Hesionidae | 0.00 | **1.30** | 14.22 | 36.12 | Phoxocephalidae | 0.50 | **1.40** | 16.98 |
| | | Capitellidae | 0.58 | **1.00** | 7.98 | | Lysianassidae | **1.77** | 1.07 | 12.52 |
| | | Aphroditidae | 0.38 | **0.80** | 7.59 | | Photidae | 1.00 | **1.40** | 10.72 |
| | SEF 1 | | | | | | | | | |
| | Polychaetes | | | | | Amphipods | | | | |
| | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) |
| October'02 | 43.49 | Sabellidae | 0.49 | **1.21** | 7.44 | 51.99 | Photidae | 0.15 | **1.03** | 11.24 |
| | | Maldanidae | 1.33 | **2.05** | 6.62 | | Lysianassidae | 0.96 | **1.88** | 10.75 |
| | | Onuphidae | 0.49 | **1.19** | 6.51 | | Urothoidae | **1.61** | 1.03 | 9.80 |
| October'03 | 43.24 | Capitellidae | **2.44** | 0.68 | 11.51 | 69.02 | Lysianassidae | 0.60 | **1.20** | 10.32 |
| | | Maldanidae | 1.18 | **2.28** | 7.43 | | Photidae | 0.00 | **0.97** | 10.06 |
| | | Sabellidae | 0.51 | **1.38** | 6.35 | | Ampeliscidae | 0.35 | **1.13** | 9.88 |
| October'04 | 43.38 | Cirratulidae | **1.40** | 0.57 | 8.02 | 52.81 | Lysianassidae | **1.26** | 0.90 | 10.70 |
| | | Orbiniidae | 0.73 | **1.02** | 6.34 | | Maeridae | **0.93** | 0.27 | 10.33 |
| | | Nereididae | **0.90** | 0.13 | 6.25 | | Photidae | 0.60 | **1.03** | 10.19 |
| October'05 | 49.30 | Capitellidae | **3.69** | 0.74 | 18.07 | 84.28 | Lysianassidae | 0.11 | **1.28** | 19.00 |
| | | Spionidae | **1.24** | 1.18 | 6.53 | | Photidae | 0.00 | **1.24** | 18.44 |
| | | Nereididae | **1.44** | 0.34 | 6.50 | | Ampeliscidae | 0.00 | **1.13** | 18.03 |
| June'06 | 71.44 | Glyceridae | 0.37 | **1.24** | 8.89 | 77.91 | Urothoidae | **1.07** | 0.83 | 16.06 |
| | | Maldanidae | 0.00 | **0.97** | 7.94 | | Lysianassidae | 0.20 | **0.96** | 15.98 |
| | | Paraonidae | 0.95 | **1.14** | 7.15 | | Ampeliscidae | 0.00 | **0.64** | 9.11 |

**Table 7.6** Continued

| | SEF 1 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | | | | Amphipods | | | | |
| | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) |
| June'07 | 44.85 | Maldanidae | 0.84 | **1.84** | 7.17 | 54.85 | Photidae | 0.60 | **1.39** | 9.92 |
| | | Orbiniidae | **1.81** | 0.92 | 6.87 | | Lysianassidae | 1.01 | **1.37** | 9.18 |
| | | Glyceridae | 0.65 | **1.37** | 6.17 | | Cheirocratidaae | 0.11 | **0.87** | 9.14 |
| June'08 | 39.13 | Nereididae | **1.74** | 0.67 | 8.44 | 53.01 | Photidae | 0.28 | **1.11** | 10.15 |
| | | Orbiniidae | **1.95** | 1.00 | 7.19 | | Cheirocratidaae | 0.11 | **0.92** | 9.06 |
| | | Dorvilleidae | 0.27 | **0.92** | 6.22 | | Caprellidae | **1.14** | 0.78 | 8.88 |
| June'09 | 41.31 | Capitellidae | **1.70** | 0.71 | 8.69 | 64.89 | Photidae | **1.01** | 0.59 | 10.68 |
| | | Orbiniidae | **1.92** | 0.77 | 7.63 | | Caprellidae | **0.76** | 0.52 | 8.75 |
| | | Nereididae | **1.71** | 0.73 | 7.04 | | Lysianassidae | **1.14** | 1.08 | 8.62 |
| | SEF 2 | | | | | | | | | |
| | Polychaetes | | | | | Amphipods | | | | |
| | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) |
| June'01 | 41.55 | Eunicidae | 0.62 | **1.39** | 7.40 | 47.48 | Photidae | **1.46** | 0.84 | 11.05 |
| | | Syllidae | 0.50 | **0.93** | 6.59 | | Urothoidae | **1.74** | 1.06 | 8.96 |
| | | Opheliidae | **1.01** | 0.70 | 6.07 | | Ampeliscidae | **1.22** | 0.87 | 8.09 |
| June'02 | 52.32 | Syllidae | 0.75 | **0.77** | 6.24 | 69.29 | Urothoidae | **1.16** | 0.82 | 14.49 |
| | | Eunicidae | **1.13** | 0.88 | 6.23 | | Lysianassidae | **0.92** | 0.68 | 11.81 |
| | | Opheliidae | **0.96** | 0.28 | 5.96 | | Maeridae | 0.31 | **0.55** | 8.93 |
| June'03 | 46.98 | Syllidae | 0.67 | **1.20** | 6.93 | 51.03 | Urothoidae | 1.43 | **1.05** | 10.04 |
| | | Eunicidae | 0.32 | **1.27** | 6.80 | | Photidae | 0.64 | **0.69** | 9.37 |
| | | Lumbrineridae | 0.32 | **1.09** | 5.94 | | Caprellidae | 0.45 | **0.99** | 9.20 |

**Table 7.6** Continued

| | SEF 2 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | | | | Amphipods | | | | |
| | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) | Avg Dissim (%) | Family | Avg Abund Im | Co | Contrib (%) |
| June'04 | 52.25 | Capitellidae | **1.13** | 0.78 | 6.61 | 71.74 | Urothoidae | 0.77 | **1.04** | 12.80 |
| | | Lumbrineridae | 0.93 | **1.01** | 6.56 | | Ampeliscidae | 0.27 | **0.95** | 11.62 |
| | | Dorvilleidae | **1.07** | 0.59 | 6.36 | | Phoxocephlidae | 0.50 | **0.77** | 10.33 |
| June'05 | 51.62 | Maldanidae | 0.66 | **1.82** | 8.06 | 65.06 | Caprellidae | 0.00 | **0.80** | 9.43 |
| | | Capitellidae | **1.76** | 0.89 | 7.59 | | Urothoidae | 0.97 | **1.04** | 9.12 |
| | | Dorvilleidae | **1.34** | 0.65 | 6.97 | | Photidae | 0.37 | **0.84** | 9.09 |
| June'06 | 69.39 | Glyceridae | 0.64 | **1.82** | 10.05 | 66.78 | Urothoidae | **1.94** | 1.21 | 16.51 |
| | | Paraonidae | 1.56 | **1.69** | 9.23 | | Lysianassidae | 0.98 | **1.47** | 13.70 |
| | | Syllidae | 0.41 | **1.57** | 8.05 | | Ampeliscidae | 0.48 | **0.93** | 9.99 |
| June'07 | 57.66 | Maldanidae | 0.84 | **3.71** | 14.25 | 57.23 | Lysianassidae | 1.28 | **2.53** | 12.75 |
| | | Paraonidae | 2.24 | **2.61** | 9.03 | | Photidae | 0.79 | **2.24** | 12.14 |
| | | Orbiniidae | **1.83** | 1.23 | 7.10 | | Urothoidae | **2.11** | 1.53 | 12.05 |
| June'08 | 52.61 | Paraonidae | **5.91** | 3.33 | 12.19 | 63.03 | Lysianassidae | 1.12 | **2.07** | 11.14 |
| | | Dorvilleidae | **2.04** | 1.34 | 7.11 | | Photidae | 0.12 | **1.49** | 10.97 |
| | | Capitellidae | **2.62** | 0.79 | 6.90 | | Cheirocratidaae | 0.39 | **1.30** | 9.19 |
| June'09 | 50.57 | Dorvilleidae | **5.88** | 1.87 | 14.56 | 71.47 | Lysianassidae | 1.36 | **1.62** | 11.31 |
| | | Capitellidae | **3.45** | 1.00 | 8.65 | | Urothoidae | 0.82 | **0.90** | 11.26 |
| | | Orbiniidae | **3.12** | 1.26 | 8.13 | | Maeridae | 0.37 | **1.22** | 10.46 |

For the SEF 2, SIMPER analysis of polychaete family abundance showed high dissimilarity (c. 69 %) between the impacted and control plots in June'06 (Table 7.6). The polychaetes that contributed most to this dissimilarity (i.e. Glyceridae, Paraonidae, and Syllidae) were less abundant at the impacted plot compared to the control plots. For amphipod family abundance, SIMPER indicated high dissimilarity between the SEF 2 impacted and control plots in June'02 (c. 69 %), June'04 (c. 72 %), June'05 (c. 65 %), June'06 (c. 67 %), and June'09 (c. 72 %) (Table 7.6). The amphipods that contributed most to this dissimilarity (see Table 7.6) were, in general, less abundant at the impacted plot compared to the control plots. The dissimilarity in polychaete and amphipod family abundance between the SEF 2 impacted and control plots was low (respectively c. 42 - 58%, & c. 47 - 57%) during the rest of the study period (respectively June'02-June'05 & June'07-June'09, and June'01, June'03 & June'07), while capitellids contributed to c. 7 – 8 % of this dissimilarity in June'04, June'05, June'08, and June'09 (Table 7.6).

Post hoc three-factor, asymmetrical, univariate PERMANOVA indicated significant difference for 'Im-vs-Co x Ti' in the abundance of: (i) Capitellidae, Hesionidae, Maldanidae, Lysianassidae, and Phoxocephalidae at the NEF ($p < 0.0001$ except $p_{Lysianassidae} < 0.05$); (ii) Ampeliscidae ($p < 0.01$), Glyceridae ($p < 0.001$), Maldanidae ($p < 0.0001$), Photidae ($p < 0.05$), and Urothoidae ($p < 0.05$) at the SEF 1; and (iii) Lysianassidae ($p < 0.001$), Photidae ($p < 0.01$), and Phoxocephalidae ($p < 0.001$) at the SEF 2 (Table 7.7).

*A posteriori* pair-wise comparisons showed that the abundance of Capitellidae at the NEF impacted plot was significantly high in November'01 ($p < 0.01$), January'03 ($p < 0.05$), and November'03 ($p < 0.01$); and significantly low in May'08 ($p < 0.01$) and April'09 ($p < 0.05$); compared to the NEF control plots in the same periods (Appendix 5). Pair-wise tests also showed significantly low abundance of: (i) Hesionidae in November'01-April'09 (except in April'03); (ii) Maldanidae in April'02-March'04, November'05, and May'08; (iii) Lysianassidae in April'03, March'04, November'05, and May'08; and (iv) Photidae in November'01-April'09 (except in November'04); at the NEF impacted plot compared with the NEF control plots in the same periods (see Appendix 5). At the SEF 1, pair-wise tests showed that the abundance of: (i) Glyceridae in June'06 and June'07 ($p < 0.01$); (ii) Maldanidae in October'02, June'06,

**Table 7.7** Results of three-factor, univariate, asymmetrical post hoc PERMANOVA for the polychaete and amphipod taxa that contributed most (in terms of number of individuals) to the high dissimilarity of samples between impacted and control plots from the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1) and southeastern 'Farm 2' (SEF 2) during the study period. The level of significance was set at 0.05. Df = Degrees of freedom, RES = Residual, Ampeliscidae (Amp), Capitellidae (Cap), Caprellidae (Capr), Dorvilleidae (Dor), Glyceridae (Gly), Hesionidae (Hes), Lysianassidae (Lys), Maeridae (Mae), Maldanidae (Mal), Paraonidae (Par), Photidae (Pho), Phoxocephalidae (Phox), Syllidae (Syl), Urothoidae (Uro), ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

| | | NEF | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Polychaetes | | | | Amphipods | | | |
| Source of Variation | df | Cap | Dor | Hes | Mal | Par | Lys | Pho | Uro |
| Impact-vs-Control = Im-vs-Co | 1 | ns | ns | *** | ** | ns | ns | **** | ns |
| Time = Ti | 13 | ns | ns | ns | ns | ns | * | ns | ns |
| Control = Co | 1 | ns | ns | ns | ** | ns | ns | ns | * |
| Im-vs-Co x Ti | 13 | **** | ns | **** | **** | ns | * | **** | ns |
| Co x Ti | 13 | ns | ns | *** | ns | **** | ns | **** | ** |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 42 | ns | * | ns | ns | **** | ns | ns | **** |
| Site(Co x Ti) = Si(Co x Ti) | 84 | ns | ns | ns | ns | ns | **** | * | ns |
| RES: Si(Im-vs-Co x Ti) | 112 | | | | | | | | |
| RES: Si(Co x Ti) | 224 | | | | | | | | |
| Total | 503 | | | | | | | | |

**Table 7.7** Continued

| SEF 1 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Polychaetes | | | | Amphipods | | | | |
| Source of Variation | df | Cap | Gly | Mal | Par | Amp | Capr | Lys | Pho | Uro |
| Impact-vs-Control = Im-vs-Co | 1 | ns | * | * | ns | ** | ns | ns | ** | ns |
| Time = Ti | 7 | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Control = Co | 1 | * | ns | ns | ns | ns | ns | ** | ns | ns |
| Im-vs-Co x Ti | 7 | ns | *** | **** | ns | ** | ns | ns | * | * |
| Co x Ti | 7 | ns | * | ns | **** | * | ns | * | ** | * |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 16 | ns | ns | ns | ** | ns | **** | ** | * | ns |
| Site(Co x Ti) = Si(Co x Ti) | 32 | ns | * | **** | ns | ns | ns | ns | *** | * |
| RES: Si(Im-vs-Co x Ti) | 48 | | | | | | | | | |
| RES: Si(Co x Ti) | 96 | | | | | | | | | |
| Total | 215 | | | | | | | | | |

| SEF 2 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Polychaetes | | | Amphipods | | | | | | |
| Source of Variation | df | Gly | Par | Syl | Amp | Capr | Lys | Mae | Pho | Phox | Uro |
| Impact-vs-Control = Im-vs-Co | 1 | ns | ns | ns | ns | ns | ns | ns | * | ns | ns |
| Time = Ti | 8 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Control = Co | 1 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| Im-vs-Co x Ti | 8 | ns | ns | ns | ns | ns | *** | ns | ** | *** | ns |
| Co x Ti | 8 | **** | *** | * | **** | ns | **** | ns | ** | ns | ** |
| Site(Im-vs-Co x Ti) = Si(Im-vs-Co x Ti) | 27 | *** | **** | **** | ** | *** | ** | * | *** | ns | *** |
| Site(Co x Ti) = Si(Co x Ti) | 54 | * | *** | *** | * | **** | * | *** | **** | *** | **** |
| RES: Si(Im-vs-Co x Ti) | 72 | | | | | | | | | | |
| RES: Si(Co x Ti) | 144 | | | | | | | | | | |
| Total | 323 | | | | | | | | | | |

and June'07 ($p < 0.05$ except $p_{June'07} < 0.01$); and (iii) Ampeliscidae and Photidae in October'03 ($p < 0.05$) and October'05-June'07 ($p < 0.001$ except $p_{Ampeliscidae\ June'06} < 0.01$ & $p_{Ampeliscidae\ June'07} < 0.05$; was significantly low at the impacted plot compared to the control plots in the same periods; while the abundance of Urothoidae at the impacted and control plots was not significantly different following the tuna penning activities (October'03-June'09) (Appendix 5). At the SEF 2, pair-wise tests showed significantly low abundance of Lysianassidae in June'07, Photidae in June'06-June'08, and Phoxocephalidae in June'06 and June'07; at the impacted plot compared with the control plots in the same period (Appendix 5).

Post hoc asymmetrical, univariate, PERMANOVA showed no significant difference in the abundance of: (i) Dorvilleidae, Paraonidae, and Urothoidae at the NEF; (ii) Capitellidae, Paraonidae, Caprellidae, and Lysianassidae at the SEF 1; and (iii) Glyceridae, Paraonidae, Syllidae, Ampeliscidae, Caprellidae, Maeridae, and Urothoidae at the SEF 2; for 'Im-vs-Co x Ti' (Table 7.7).

### 7.3.2(iii) Relationship between sediment attributes and macroinvertebrates

The BEST analysis indicated significant correlation between: (i) values of the BOPA-FF index ($\rho = 0.371$, $p < 0.001$) and M-AMBI ($\rho = 0.300$, $p < 0.01$) and sediment POCC; and (ii) polychaete ($\rho = 0.335$, $p < 0.001$) and amphipod ($\rho = 0.462$, $p < 0.001$) family abundance and a combination of MSGS and POCC in sediment; at the NEF impacted plot; and between polychaete ($\rho = 0.200$, $p < 0.01$) and amphipod ($\rho = 0.192$, $p < 0.01$) family abundance and respectively combinations of MSGS and POCC in sediment, and MSGS, and POCC and PONC in sediment, at the NEF control plots, overall, during the study period (Table 7.8). The BEST analysis also indicated significant correlation, overall, at the NEF impacted and control plots between: (i-a) BOPA-FF ($\rho = 0.594$, $p < 0.01$), M-AMBI ($\rho = 0.779$, $p < 0.01$) and amphipod family abundance ($\rho = 0.839$, $p < 0.001$) and sediment POCC, and (i-b) polychaete family abundance and a combination of MSGS and POCC in sediment ($\rho = 0.702$, $p < 0.001$), in November'01; (ii-a) M-AMBI ($\rho = 0.779$, $p < 0.01$) and polychaete ($\rho = 0.702$, $p < 0.001$) family abundance and PONC in sediment, and (ii-b) amphipod family abundance and a combination of MSGS and PONC in sediment ($\rho = 0.548$, $p < 0.01$), in April'02; (iii) amphipod family abundance and a combination of MSGS, and POCC

and PONC in sediment ($\rho = 0.496$, $p < 0.05$) in January'03; (iv-a) values of the BOPA-FF index and a combination of MSGS and PONC in sediment ($\rho = 0.616$, $p < 0.05$), and (iv-b) values of the M-AMBI index ($\rho = 0.610$, $p < 0.01$) and polychaete ($\rho = 0.376$, $p < 0.05$) family abundance and PONC in sediment, in April'03; and (v) BOPA-FF ($\rho = 0.611$, $p < 0.01$), values of the M-AMBI index ($\rho = 0.499$, $p < 0.05$) and polychaete family abundance ($\rho = 0.410$, $p < 0.05$) and PONC in sediment, in November'03. No significant correlation was indicated between sediment physico-chemical variables and attributes of the macroinvertebrate assemblages, overall, at the NEF impacted and control plots before initiation of the tuna penning activities (November'00 & March'01) and afterwards in March'04-April'09 (Table 7.8).

At the SEF 1, BEST analysis showed significant correlation between: (i) values of the BOPA-FF index ($\rho = 0.305$, $p < 0.01$) and amphipod ($\rho = 0.353$, $p < 0.01$) family abundance and a combination of MSGS, and POCC and PONC in sediment; (ii) values of the M-AMBI index and a combination of POCC and PONC in sediment ($\rho = 0.156$, $p < 0.05$); and (iii) polychaete family abundance and a combination of MSGS and PONC in sediment ($\rho = 0.275$, $p < 0.05$), at the control plots overall during the study period (Table 7.8). No significant correlation was indicated between sediment physico-chemical variables and attributes of the macroinvertebrate assemblages, overall, at the SEF 1 impacted plot during the study period. No significant correlation was indicated overall at the SEF 1 impacted and control plots between: (i) polychaete family abundance and POCC ($\rho = 0.723$, $p < 0.001$) in October'02; (ii) values of the BOPA-FF index and POCC in sediment ($\rho = 0.654$, $p < 0.05$), in October'03; (iii) polychaete family abundance and PONC in sediment ($\rho = 0.589$, $p < 0.05$), in October'04; (iv-a) values of the BOPA-FF index ($\rho = 0.591$, $p < 0.05$) and polychaete family abundance ($\rho = 0.723$, $p < 0.001$) and POCC in sediment, and (iv-b) values of the M-AMBI index and PONC in sediment ($\rho = 0.657$, $p < 0.05$), in October'05; (v-a) values of the BOPA-FF index and POCC in sediment ($\rho = 0.541$, $p < 0.05$), (v-b) values of the M-AMBI index and a combination of POCC and PONC in sediment ($\rho = 0.705$, $p < 0.01$), (v-c) polychaete family abundance and a combination of MSGS, and POCC and PONC in sediment ($\rho = 0.738$, $p < 0.001$), and (v-d) amphipod family abundance and a combination of MSGS and POCC in sediment($\rho = 0.534$, $p < 0.05$), in June'06; and (vi-a) values of the BOPA-FF index and POCC in sediment ($\rho = 0.522$, $p < 0.05$), and (vi-b) values of the M-AMBI index ($\rho = 0.559$, $p < 0.05$) and amphipod

**Table 7.8** BEST results showing the best explanatory variable (Best Exp Var) or combination of variables that explains the observed variation in polychaete and amphipod family abundance at the impacted and control plots overall during the study period, and overall at the impacted and control plots during each sampling date, at the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1) and southeastern 'Farm 2' (SEF 2). Level of significance set at 0.05. ρ-value = Pearson's correlation coefficient, MSGS = mean sediment grain size, POCC = percent organic carbon content, PONC = percent organic nitrogen content, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| | NEF | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | BOPA-FF | | M-AMBI | | Polychaete Family-abundance | | Amphipod Family-abundance | |
| | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| Impact | 0.371, *** | POCC | 0.300, ** | POCC | 0.335, *** | MSGS, POCC | 0.462, *** | MSGS, POCC |
| Control | 0.074, ns | POCC, PONC | 0.077, ns | MSGS | 0.200 ** | MSGS, POCC | 0.192, ** | MSGS, POCC, PONC |
| November'00 | 0.340, ns | MSGS, POCC, PONC | 0.224, ns | POCC | 0.024, ns | MSGS | 0.215, ns | MSGS, POCC |
| March'01 | 0.167, ns | MSGS, PONC | 0.118, ns | POCC | 0.054, ns | MSGS | 0.019, ns | MSGS, PONC |
| November'01 | 0.594, ** | POCC | 0.779, ** | POCC | 0.702, *** | MSGS, POCC | 0.838, *** | POCC |
| April'02 | 0.325, ns | MSGS | 0.544, * | PONC | 0.537, * | PONC | 0.548, ** | MSGS, PONC |
| January'03 | 0.456, ns | MSGS, POCC | 0.323, ns | PONC | 0.369, ns | PONC | 0.496, * | MSGS, POCC, PONC |
| April'03 | 0.616, * | MSGS, PONC | 0.610, ** | PONC | 0.376, * | PONC | 0.442, ns | MSGS, PONC |
| November'03 | 0.611, ** | PONC | 0.499, * | PONC | 0.410, * | PONC | 0.459, ns | MSGS, POCC, PONC |
| March'04 | 0.206, ns | POCC, PONC | 0.134, ns | POCC | 0.222, ns | POCC | 0.171, ns | POCC |
| November'04 | -0.040, ns | POCC | -0.101, ns | POCC | 0.218, ns | PONC | 0.154, ns | POCC |
| November'05 | 0.149, ns | MSGS, POCC, PONC | 0.066, ns | MSGS, PONC | 0.071, ns | POCC | 0.261, ns | MSGS, POCC, PONC |
| April'06 | 0.569, ns | MSGS, POCC, PONC | 0.322, ns | MSGS | 0.345, ns | POCC | 0.394, ns | MSGS, PONC |
| June'07 | 0.070, ns | PONC | 0.159, ns | POCC | 0.104, ns | POCC | 0.106, ns | POCC |
| May'08 | 0.031, ns | POCC | 0.159, ns | POCC | 0.005, ns | MSGS, POCC | -0.051, ns | POCC |
| April'09 | 0.063, ns | PONC | 0.115, ns | MSGS, POCC | -0.054, ns | POCC | 0.087, ns | MSGS |

**Table 7.8** Continued

| | SEF 1 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | BOPA-FF | | M-AMBI | | Polychaete Family-abundance | | Amphipod Family-abundance | |
| | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| Impact | 0.270, ns | POCC | 0.144, ns | POCC, PONC | 0.054, ns | POCC | 0.136, ns | POCC, PONC |
| Control | 0.305, ** | MSGS, POCC, PONC | 0.156, * | POCC, PONC | 0.275, * | MSGS, PONC | 0.353, ** | MSGS, POCC, PONC |
| October'02 | 0.299, ns | MSGS | 0.221, ns | POCC | 0.723, *** | POCC | 0.372, ns | POCC, PONC |
| October'03 | 0.654, * | POCC | 0.439, ns | POCC | 0.298, ns | POCC | 0.391, ns | POCC |
| October'04 | 0.367, ns | MSGS, PONC | 0.345, ns | PONC | 0.589, * | PONC | 0.325, ns | PONC |
| October'05 | 0.591, * | POCC | 0.657, * | PONC | 0.723, *** | POCC | 0.552, ns | POCC |
| June'06 | 0.541, * | POCC | 0.705, ** | POCC, PONC | 0.738, *** | MSGS, POCC, PONC | 0.534, * | MSGS, POCC |
| June'07 | 0.522, * | POCC | 0.559, * | MSGS, POCC, PONC | 0.264, ns | MSGS, PONC | 0.434, ** | MSGS, POCC, PONC |
| June'08 | 0.310, ns | MSGS, POCC, PONC | 0.058, ns | POCC | 0.396, ns | PONC | 0.410, ns | MSGS, POCC, PONC |
| June'09 | 0.209, ns | POCC | 0.081, ns | POCC | -0.001, ns | POCC | 0.419, ns | POCC |
| | SEF 2 | | | | | | | |
| | BOPA-FF | | M-AMBI | | Polychaete Family-abundance | | Amphipod Family-abundance | |
| | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| Impact | 0.256, * | PONC | 0.048, ns | MSGS, POCC | -0.035, ns | POCC | 0.221, * | MSGS |
| Control | 0.251, *** | MSGS, PONC | 0.066, ns | PONC | 0.114, ns | MSGS, POCC | 0.288, *** | MSGS, POCC |
| June'01 | 0.393, ns | MSGS, POCC, PONC | 0.454, * | MSGS, POCC, PONC | 0.321, ns | MSGS, POCC | 0.580, ** | MSGS, POCC, PONC |
| June'02 | -0.114, ns | MSGS | 0.066, ns | POCC | 0.167, ns | MSGS, POCC | 0.220, ns | POCC |
| June'03 | 0.376, ns | POCC, PONC | -0.004, ns | MSGS | 0.047, ns | POCC | 0.422, ns | MSGS, POCC, PONC |

**Table 7.8** Continued

| | SEF 2 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | BOPA-FF | | M-AMBI | | Polychaete Family-abundance | | Amphipod Family-abundance | |
| | ρ-value | Best Exp Var- | ρ-value | Best Exp Var | ρ-value | Best Exp Var | ρ-value | Best Exp Var |
| June'04 | 0.355, ns | POCC | 0.186, ns | POCC | 0.060, ns | MSGS | 0.201, ns | MSGS, POCC, PONC |
| June'05 | 0.086, ns | MSGS, POCC | 0.176, ns | MSGS | 0.213, ns | MSGS, PONC | 0.403, * | POCC, PONC |
| June'06 | 0.647, *** | MSGS, POCC, PONC | 0.687, *** | MSGS, PONC | 0.690, *** | MSGS, PONC | 0.597, *** | MSGS, POCC, PONC |
| June'07 | 0.415, ns | POCC | 0.441, ns | POCC | 0.616, *** | POCC | 0.649, *** | POCC |
| June'08 | 0.263, ns | POCC, PONC | 0.640, ** | POCC | 0.588, *** | POCC | 0.648, *** | POCC |
| June'09 | 0.049, ns | POCC | 0.137, ns | POCC | 0.216, ns | MSGS | 0.300, ns | POCC |

family abundance ($\rho = 0.434$, $p < 0.01$) and a combination of MSGS, and POCC and PONC in sediment, in June'07. No significant correlation was indicated between sediment physico-chemical variables and attributes of the macroinvertebrate assemblages, overall, at the SEF 1 impacted and control plots in June'08 and June'09 (Table 7.8).

At the SEF 2, BEST analysis indicated significant correlation between values of the BOPA-FF index and PONC in sediment ($\rho = 0.256$, $p < 0.05$), and amphipod family abundance and MSGS ($\rho = 0.221$, $p < 0.05$), at the impacted plot; and between values of the BOPA-FF index and a combination of MSGS and PONC in sediment ($\rho = 0.251$, $p < 0.001$), and amphipod family abundance and a combination of MSGS and POCC in sediment ($\rho = 0.288$, $p < 0.001$), at the control plots, overall, during the study period (Table 7.8). A significant correlation was indicated, overall, at the SEF 2 impacted and control plots between: (i) values of the M-AMBI index ($\rho = 0.454$, $p < 0.05$) and amphipod family abundance ($\rho = 0.580$, $p < 0.01$) and a combination of MSGS, and POCC and PONC in sediment, in June'01; (ii) amphipod family abundance and a combination of POCC and PONC in sediment ($\rho = 0.403$, $p < 0.05$) in June'05; (iii-a) values of the BOPA-FF index ($\rho = 0.647$, $p < 0.001$) and amphipod family abundance ($\rho = 0.597$, $p < 0.001$) and a combination of MSGS, and POCC and PONC in sediment, and (iii-b) values of the M-AMBI index ($\rho = 0.687$, $p < 0.001$) and polychaete family abundance ($\rho = 0.690$, $p < 0.001$) and a combination of MSGS and PONC in sediment, in June'06; (iv) polychaete ($\rho = 0.616$, $p < 0.001$) and amphipod ($\rho = 0.649$, $p < 0.001$) family abundance and POCC in sediment, in June'07; and (v) values of the M-AMBI index ($\rho = 0.640$, $p < 0.01$) and polychaete ($\rho = 0.588$, $p < 0.001$) and amphipod family abundance ($\rho = 0.648$, $p < 0.001$) and POCC in sediment, in June'08 (Table 7.8).

## 7.4 Discussion

The present study assessed for the potential presence of a temporal pattern in attributes of soft bottom benthic assemblages present in the vicinity of three tuna farms located off the northeastern to southeastern coast of Malta, over a period of ten years of tuna penning. Data on sediment physico-chemical attributes and macroinvertebrate assemblages recorded from three different ABT farms and six reference areas before initiation of the tuna penning activities and thereafter at six-monthly or annual

intervals, was analysed using standard statistical techniques and benthic biotic indices; the latter being currently used in Europe to classify the ecological status of coastal waters as per the EU's Water Framework Directive (WFD). As far as the present author is aware, this is the first time that a study on the influence of tuna penning activities on macroinvertebrate assemblages present in the vicinity of tuna farms incorporates such a complete set of data, and which aims to assess temporal patterns of benthic disturbance caused by the repeated use of an ABT aquaculture site for any region worldwide.

The recovery of macroinvertebrate assemblages following fish farm abatement depends on farm characteristics as well as local biotic, environmental, and oceanographic factors (Salvo *et al*., 2017). The three ABT farms included in the present study differed in size and feed management regime, as well as in the factors that characterise the marine environment in their vicinity (Mangion *et al*., 2017, 2018); hence, one would expect differences in the temporal pattern of influence of the tuna ranching activities among the three ABT farms during the ten-year study period. The disturbance to benthic habitat caused by fish farming activities is reduced when the amount of uneaten fish-feed that accumulates on the sediment below fish cages is minimised (e.g. Abdou *et al*., 2017; Ballester-Moltó, Sanchez-Jerez, Cerezo-Valverde, & Aguado-Giménez, 2017; Tomassetti *et al*., 2016). Slow recovery rates at sites used repeatedly for aquaculture (Keeley *et al*., 2015) are observed when organic matter accumulates on the seabed (Brooks *et al*., 2004) and continues to release nutrients after fish farm abatement, hindering complete benthic recovery (e.g. Karakassis *et al*., 1999).

In the present study, differences in the fallowing history (Macleod *et al*., 2008; Tomassetti *et al*., 2009), stocking density, and feed input (Edgar *et al*., 2010) of particular cages within the same farm resulted in significant small scale variation in the temporal pattern in values of the BOPA-FF and M-AMBI indices, and in dispersion of samples of polychaete and amphipod family abundance, namely among the seabed areas occupied by the individual cages within the same farm, and over and above the expected variation among the different ABT farms. The high variability in attributes of macrofaunal assemblages between different sites at fish farms compared with reference areas (e.g. Fernandez-Gonzalez *et al*., 2013;

Mangion *et al*., 2014) is characteristic of stressed assemblages (e.g. Stark *et al*., 2003; Warwick & Clarke, 1993).

For the same ABT farms considered in the present study, Mangion *et al*. (2018) reported a higher magnitude of influence at the tuna farm located off the northeastern coast of Malta – which is the largest farm in terms of holding capacity – compared with the other two farms located off the southeastern coast of the island. These results corroborate the expectation that benthic ecological quality will be lower at fish farm sites having a higher total annual production (Borja *et al*., 2009b). Mangion *et al*. (2014) reported a significant increase in levels of POCC and PONC in sediment, and an elevated abundance of capitellid polychaetes in the vicinity of the NEF tuna cages following the first fish farm production cycle (November'01), which appeared to have resulted from the uneaten feed-fish accumulated on the sediment (Holmer *et al*., 2008); such results are similar to the present findings. The present results also show significant differences in multivariate sediment physico-chemical, polychaete family abundance and amphipod family abundance data, and in values of the BOPA-FF and M-AMBI indices at the NEF impacted plot, where 'Bad' (BOPA-FF) and 'Poor' (M-AMBI) benthic ecological quality was recorded in the same period. The variation in values of the BOPA-FF and M-AMBI indices, and in macroinvertebrate family abundance recorded overall at the NEF impacted and control plots was significantly influenced by the sediment POCC in the same period (November'01-November'03). Such results are in concordance with previous findings at other study areas affected by various environmental disturbances (De-la-Ossa-Carretero *et al*., 2016) including aquaculture (e.g. Bouchet & Sauriau, 2008; Mangion *et al*., 2017, 2018).

The observed changes in sediment physico-chemical attributes and attributes of the benthic assemblages below the cages of the NEF were conspicuous during autumn, towards the end of the tuna penning season. It would seem that, following tuna farm abatement in winter, the uneaten feed-fish accumulated on the sediment underneath the tuna pens start to decompose, and only fish bones and other organic matter persist on the sediment (Mangion *et al*., 2014). Storms and bottom currents disperse this organic matter, and there is some improvement in sediment quality

and in attributes of the associated macroinvertebrate assemblages (e.g. Mangion *et al*., 2014; Marin *et al*., 2007; Vita & Marin, 2007). These observations are in concordance with the findings from studies on the environmental influence of other types of Mediterranean fish farms that report decreased sediment organic matter content below fish cages following fish farm abatement (e.g. Karakassis *et al*., 1998; Neofitou *et al*., 2010; Pohle *et al*., 2001).

Recovery of macrobenthic assemblages from organic enrichment is indicated by changes in the composition of the infaunal community. While polychaetes contribute most of the total macroinvertebrate abundance during the first month of recovery, increased abundance of sensitive taxa and decreased abundance of opportunistic taxa are observed during the fallow period (e.g. Brooks *et al*., 2004; Karakassis *et al*., 1999; Keeley *et al*., 2014; Lu & Wu, 1998; Macleod *et al*., 2007; Salvo *et al*., 2017; Zhulay *et al*., 2015). Proximate fish farm production or fallow sites may act as reservoirs of opportunists that are capable of rapid dispersal and re-establishment of high population densities (Keeley *et al*., 2015; Valdemarsen, Hansen, Ervik, & Bannister, 2015), while recolonisation by amphipods is also dependent on input from fouling communities (Fernandez-Gonzalez, Martinez-Garcia, & Sanchez-Jerez, 2016). Water mass stratification events and higher temperatures in summer may hinder the dispersal of opportunistic species from adjacent sites (Salvo *et al*., 2017), while low temperatures and low recruitment may also explain the slow recovery observed during fallow periods (Brooks *et al*., 2004). Full recovery of the sediment following each fish farm production cycle is not crucial for limiting significant adverse influences on the benthic assemblages, as long as the sediment recovers sufficiently from fish farming activities to withstand additional organic loading without accumulative damage (Macleod *et al*., 2007).

In the present study, the elevated sediment POCC recorded following the first year of fish farming operations at the NEF decreased significantly in the subsequent sampling dates, and multivariate sediment physico-chemical data showed significant differences at the NEF impacted plot compared with the latter half of the study period. Dorvilleid polychaetes; which are known indicators of benthic recovery at aquaculture sites (Aguado-Giménez *et al*., 2007); appeared in elevated (albeit not significant)

abundance at the NEF impacted plot in November'05, when 'Poor' benthic ecological quality was recorded. Values of the BOPA-FF and M-AMBI indices at the NEF impacted plot indicated significantly lower benthic ecological quality in the first half of the study (November'01, November'03, & November'05) compared with the 'High' or 'Good' EQS categorisations recorded thereafter (April'06-April'09), while multivariate polychaete and amphipod family abundance differed significantly at this plot compared with successive sampling dates and showed increased similarity to reference conditions towards the end of the study period. Furthermore, capitellid abundance was significantly lower at the NEF impacted plot compared with the control plots (May'08 & April'09), and sediment physico-chemical attributes showed no significant influence on macroinvertebrate assemblages at the NEF (November'05-April'09) in this period. Taken together, these observations indicate that the alternate use of production and fallow periods together with an improved feed management regime at the NEF were sufficient to mitigate the adverse benthic influence of tuna penning activities during the repeated use of this site for aquaculture, and resulted in a short-term, 'pulse' disturbance.

The influence of ABT farming activities within the footprint of both southeastern farms was indicated by a significant increase in values of the BOPA-FF index, by a significant decrease in values of the M-AMBI index, by increased (albeit non significant) dissimilarity in polychaete and amphipod assemblages, and lastly by significant changes in the dispersion of samples of polychaete and amphipod family abundance during the study period. The elevated levels of sediment FFBC, POCC and PONC below the tuna cages of SEF 1 compared to SEF 2; the significantly low abundance of amphipod taxa, and the significant decrease to 'Moderate' (BOPA-FF in October'03), 'Poor' (M-AMBI in October'05) and 'Bad' (BOPA-FF in October'05) benthic ecological quality at the SEF 1 impacted plot during the first part of the study period, indicate that the magnitude of influence on benthic habitat in the immediate vicinity of SEF 2, which retained 'Good' or 'High' EQS, was not as large, in concordance with the findings by Mangion *et al*. (2018). On the other hand, the significant decrease to 'Moderate' benthic ecological quality (BOPA-FF) (June'09), and the significantly low abundance of sensitive macroinvertebrate taxa recorded within the footprint of both southeastern farms towards the end of the study period, suggested that the

cumulative effect of nutrient enrichment during the repeated use of the southeastern farm sites for aquaculture resulted in a 'press' disturbance.

A peak in levels of POCC and PONC in the sediment, and high (albeit non significant) dissimilarity in sediment physico-chemical attributes, polychaete family abundance, and amphipod family abundance data, was recorded at the two southeastern control plots at the end of the study period (June'09). Attributes of the macroinvertebrate assemblages at the southeastern control plots were significantly correlated with MSGS, and POCC and PONC in the sediment during the study period, while 'Moderate' (BOPA-FF in June'09 and M-AMBI in June'06) EQS categorisation was recorded at the control plot located 2 km away from SEF 2 towards the end of the study period. Furthermore, sediment physico-chemical attributes appeared to influence values of the BOPA-FF and M-AMBI indices, as well as polychaete and amphipod family abundance at the impacted plot toward the end of the study period at SEF 2 only. The acquired sea current data showed that the predominant sea current in the vicinity of the two southeastern farms had a southern direction (189 $^{o}$), and a mean velocity of 0.185 $ms^{-1}$. The down-current orientation of SEF 2 with respect to SEF 1 may account for the persistent influence of tuna penning at both the SEF 2 impacted and control plots, since particulate organic matter may have been transported down-current (Mangion *et al*., 2018). These observations suggest that the ABT farms located off the southeastern coast of Malta in coastal waters had an additive effect (Mangion *et al*., 2018), given that they are located relatively close to each other (only 1 km apart). The coastal waters in which these farms are located may inherently have higher nutrient loading that is typical of waters off the southeastern coast of Malta (compared to the north of the islands) (Axiak *et al*., 2000), which results from higher coastal use in the latter region (Mallia *et al*., 2002).

The significant variability in benthic ecological quality (BOPA-FF) recorded among the southeastern control plots at the end of the study period (June'09); namely 'Good' and 'Moderate' EQS; hindered detection of a significant influence of tuna penning activities within the footprint of SEF 1, even though the mean EQS classification there was 'Moderate' in that period. Significant temporal variability among control plots was also indicated for values of the M-AMBI index

and the multivariate sediment physico-chemical data, polychaete family abundance, and amphipod family abundance at each of the investigated tuna farms. Furthermore, significant variability in the temporal pattern in attributes of the macroinvertebrate assemblages among control plots was detected at the smallest spatial scale of sites (c. 100 m) at both the northeastern and southeastern study areas. These observations highlight the importance of including multiple reference areas in environmental impact monitoring studies at fish farm sites (e.g. Fernandes *et al*., 2001; Mangion *et al*., 2018).

Macroinvertebrate assemblages may undergo temporal changes within the whole study area (Edgar *et al*., 2010), while natural seasonal variability is caused by recruitment and increased food availability in summer and spring, and decreased macrofaunal abundances in winter due to hydrodynamical stress and predation pressures (Reiss & Kröncke, 2005a). Cold winters may not always have an impact on the abundance of opportunistic taxa, which largely influence the value of a given indicator taxon index (Kröncke & Reiss, 2010). Although the sensitivity of indicator taxon indices to seasonal natural variability is relatively low compared with that of diversity indices (Kröncke & Reiss, 2010; Reiss & Kröncke, 2005b), present findings showed significant seasonal variability in values of the BOPA-FF and M-AMBI indices, as well as in polychaete and amphipod family abundance recorded from control plots located 1-2 km away from the NEF before tuna penning activities commenced, while the EQS classification turned out to be lower or higher in winter depending on the index used (see Dauvin *et al*., 2007). These results contrast with previous findings of no significant variability in attributes of macroinvertebrate assemblages associated with the 'bare sand' habitat located off the coast of the Maltese Islands between summer and winter seasons (Grech Santucci, 2005). While background temporal changes in organic matter content and abundance of first-order opportunistic polychaetes (e.g. Edgar *et al*., 2010) may hinder detection of changes in macroinvertebrate assemblages under the influence of an anthropogenic disturbance (Magurran *et al*., 2010), seasonal variability in macroinvertebrate community parameters at fish farm sites is more pronounced compared to that recorded at reference areas (Neofitou *et al*., 2010), and similar trends in the benthic community are recorded between seasons, over and above seasonal variability (Chainho *et al*., 2010). In the present study, the influence of

tuna penning activities on macroinvertebrate assemblages in the immediate vicinity of the NEF was evident despite the significant seasonal variability recorded in this study area before tuna penning activities commenced.

Results from the NEF during its first years of operation indicated 'Bad' and 'Poor' benthic ecological quality, which increased significantly during the fallow periods to 'High' or 'Good' EQS, with no significant differences up to the end of the study period. It is concluded that the observed changes in the macrofaunal assemblages resulted from accumulation of uneaten feed-fish on the seabed. Feed management at the NEF improved following the first years of operation, and was sufficient to mitigate the benthic influence of the ABT farming activities during the repeated use of this site for aquaculture. On the other hand, the temporal pattern of influence at both of the SEFs reflected a 'press' disturbance over several years of farming operation. Such disturbance seems to have resulted from an additive effect of cumulative nutrient enrichment from the two southeastern farms, given that they are only 1 km apart. These results highlight the importance of monitoring of feed wastage (Ballester-Moltó *et al*., 2017) and good spatial planning of coastal aquaculture activities (Mangion *et al*., 2018) to improve sustainability, especially since most countries are moving toward establishing "Allocated Zones for Aquaculture" (AZAs) (see Sanchez-Jerez *et al*., 2016). On the other hand, further study on the response of ecological quality ratios to natural temporal variability in the applicability of indicator taxon indices for long-term environmental impact monitoring programs, is recommended (Kennedy, Arthur, & Keegan, 2011). Finally, the high spatio-temporal variation of the influence of the tuna farms on macroinvertebrate assemblages highlights the importance of including multiple impacted and reference areas as well as replicated temporal sampling in assessing the environmental influence of these activities (Mangion *et al*., 2018).

# CHAPTER 8
# GENERAL DISCUSSION

## 8.1 Introduction

Research aimed at improving the environmental sustainability of the aquaculture industry is important given the global increasing trend in aquaculture production (Valdemarsen *et al*., 2015). In Malta, the Atlantic Bluefin Tuna (ABT) penning industry has followed this trend, with total annual production increasing twentyfold since it's initiation in 2000 (Camilleri, 2017; FAO, 2005), Such an increase may potentially be reflected in increased environmental impact of tuna penning in the Maltese Islands (Valdemarsen *et al*., 2015). A major concern regarding the environmental sustainability of tuna penning is the accumulation of uneaten feed-fish on the seabed in the vicinity of fish cages, which may lead to adverese effects on benthic habitat (Aguado *et al*., 2004; Aguado-Giménez *et al*., 2006; Borg & Schembri, 2005; Vita & Marin, 2007; Vita *et al*., 2004a), and hence on the associated biotic assemblages (Borg & Schembri, 2005; Vezzulli *et al*., 2008; Vita & Marin, 2007; Vita *et al*., 2004a). Impoverishment of benthic macrofauna may result in habitat loss and decreased biodiversity, as well as decreased macrofaunal capacity to degrade organic material accumulated on the sediment (Valdemarsen *et al*., 2015). It is therefore important to conserve proper functioning of macroinvertebrate assemblages in the vicinity of fish cages in order to mitigate the potentially adverse influence of aquaculture activities on benthic habitat (Valdemarsen *et al*., 2015). In the Mediterranean and beyond, most aquaculture operations are located on a soft bottom seabed.

Workers propose that differences in the magnitude and spatial extent of influence of fish farming activities on soft bottom benthic habitat arise due to variations in farm characteristics, such as farmed species, feed management, years of operations and total annual production; and on local environmental factors, such as the sea current regime, water depth and the physico-chemical characteristics of the sediment (e.g. Borja *et al*., 2009c; Tomassetti *et al*., 2009, 2016). In the case of ABT ranches, their influence on benthic habitiat is expected to differ from that caused by other types of fish farms, such as those rearing sea bass and sea bream, due to the larger size of the ABT and the use of whole feed-fish instead of formulated feed to feed the tuna. Research has increased our understanding of the influence of ABT ranching on the surrounding marine environment (Aksu *et al*., 2010, 2016; Dal Zotto *et al*., 2016; Forrestal *et al*., 2012;

Hospido & Tyedmers, 2005; Kružić *et al*., 2014; Matijević *et al*., 2006, 2008; Šegvić Bubić *et al*., 2011; Vezzulli *et al*., 2008; Vizzini & Mazzola, 2012). However, the few available studies that consider the potential influence of tuna penning activities on benthic habitat reach different conclusions regarding the level of impact (e.g. Jahani *et al*., 2012; Marin *et al*., 2007; Moraitis *et al*., 2013; Vita & Marin, 2007). Variation in experimental designs, methods and indicators used hinder comparison of results of aquaculture monitoring studies among study areas (Kalantzi & Karakassis, 2006), while experimental designs may be undermined by a lack of consideration of the appropriate spatio-temporal scales at which the influence of aquaculture activities on the surrounding environment varies (but see Martinez-Garcia, Fernandez-Gonzalez, Aguado-Giménez, Sánchez-Lizaso, & Sanchez-Jerez, 2018). There is, therefore, the need for more detailed studies on the influence of ABT penning on benthic habitat and the associated macroinvertebrate assemblages; in particular, studies considering before-impact data, multiple tuna farms, far-field effects, and long-term patterns of influence which allow for a robust and comprehensive assessment, are lacking, while there is no general agreement on the biological indicators that best signal potential change in enviromental atttributes resulting from the activity. The need for effective aquaculture monitoring programmes and good marine environmental quality has increased since publication of the EU's Water Framework Directive (WFD) and Marine Strategy Framework Directive (MSFD) (Martinez-Garcia *et al*., 2018). More knowledge on the patterns of influence of tuna penning activities on benthic macroinvertebrate assemblages will help formulate effective environmental monitoring programmes and mitigation strategies for potential adverse influence on ecology, which are essential to improve environmental sustainability of the ABT farming industry.

The aim of the present chapter is to: (i) integrate findings from the preceding chapters on the influence of tuna penning activities on macroinvertebrate assemblages associated with the soft bottom habitat; (ii) review the current practices aimed at, and challenges in, minimising or eliminating the potentially adverse influence of aquaculture on benthic habitat; hence, enabling assessment of the implications for environmental monitoring and management of ABT ranches; and (iii) provide recommendations for further research which may help coastal managers improve the environmental sustainability of the tuna penning industry.

## 8.2 Findings on the influence of tuna penning activities on macroinvertebrate assemblages associated with the soft bottom habitat

Tuna penning is a relatively new industry, hence not much is known about its influence at the ecosystem level (but see Vezzulli *et al*., 2008). The present research work was necessary since environmental monitoring implemented to date at Maltese tuna farms, as required by the local Environmental and Resources Authority (ERA), incorporated data analysis for each separate monitoring session per individual farm; hence, collective analysis of data and integrated assessment that would help to better understand the potentially adverse environmental influence of the activity were lacking. Data on sediment quality (i.e. MSGS, POCC, & PONC) and benthic macroinvertebrate assemblages used in the present work was collected from three ABT farms and reference sites; quantitative samples were collected from the seabed at incremental distances from the sea cages (i.e. c. 0 m, 100 m, 1 km, & 2 km away) before the start of the farming operations, and after initation of the activity, at six-monthly or annual intervals, over a period of ten years. Such data set is considered unique to date in that it is probably one of the largest and most complete for any study worldwide that deals with the influence of tuna penning activities on benthic macroinvertebrate assemblages.

In Malta, Borg and Schembri (2005) showed that tuna penning activities resulted in an immediate adverse influence on macrofaunal abundance and species richness that was mostly limited to the area where uneaten feed-fish had accumulated on the sediment below the cages. The deterioration of macrobenthic assemblages in the vicinity of tuna ranches can be prevented given a good understanding of the influence of tuna penning activities on the soft bottom habitat, which in turn allows for implementation of appropriate mitigation strategies to minimise or eliminate the potentially adverse effects of the activity. The present research was therefore aimed at examining the various aspects of potential influence of tuna penning activities on benthic macroinvertebrate assemblages, namely, whether:

a) there is an immediate impact of tuna penning activities on macrobenthic assemblages and signs of recovery of the benthic habitat following abatement of the farming activities in winter (Chapter 2);
b) polychaete, mollusc, amphipod, and decapod taxocenes act as good indicators of benthic change resulting from ABT farming activities (Chapter 3);
c) there are differences in the magnitude and spatial extent of influence on benthic habitat resulting from tuna farms that differ in size, in the adopted feed management strategy, and in their location (Chapter 4);
d) there is a spatial pattern in influence on attributes of macronvertebrate assemblages with incremental distance (0 m, 100 m, 1 km, and 2 km) from the tuna pens (Chapter 5);
e) indices developed under the WFD (i.e. AMBI, BENTIX, BOPA, BOPA-Fish farming index, and M-AMBI) are suitable for use in aquaculture monitoring studies in the Maltese Islands (Chapter 6); and
f) the disturbance on benthic macroinvertebrate assemblages during ten years of tuna penning activities, if present, has a temporal pattern (Chapter 7).

Findings from the present research is expected to help determine:

a) the magnitude of influence, if present, of tuna penning activities on benthic macroinvertebrate assemblages, and the effectiveness of tuna farm abatement in winter as an impact mitigation strategy (Chapter 2);
b) the specific faunal group/s that can be utilised instead of extensive biotic data used in classical detailed statistical analyses, to decrease effort in environmental monitoring studies for tuna penning (Chapter 3);
c) the appropriate spatial scale at which the potential influence of tuna penning activities on benthic macroinvertebrate assemblages should be investigated (Chapter 4);
d) whether variation in benthic biotic diversity with incremental distance from tuna farm sites concords with the Pearson-Rosenberg (P-R) (1978) model for macroinvertebrate assemblages along a gradient of increasing organic enrichment (Chapter 5); and
e) whether repeated use of tuna penning sites for aquaculture results in a ‘pulse’ or ‘press’ type of disturbance in the long term (Chapter 7).

The present research is also expected to serve as an important contribution with respect to assessment of the influence of ABT ranching on coastal ecosystems in relation to the WFD, particularly through the application of benthic biotic indices (BBIs) that have been proposed specifically for use in monitoring as required by the Directive (Chapter 6).

Results from the first study aspect ([a] above) showed a significant increase in sediment POCC and PONC, and a non-significant increase in the abundance of capitellid polychaetes in the vicinity of the fish cages towards the end of the tuna penning season (see Chapter 2); such results are in concordance with previous findings at other Mediterranean tuna farms (Jahani *et al*., 2012; Marin *et al*., 2007; Vita & Marin, 2007). The same study identified that similar results were obtained at a distance of 100-200 m away from the cages. Such spatial extent of influence of tuna penning on the benthos exceeds that reported for Mediterranean farms rearing smaller fish species, such as sea bream and sea bass (e.g. Di Marco *et al*., 2017; Karakassis *et al*., 2000, 2002; Tomassetti *et al*., 2016). Signs of recovery of the state of the sediment and associated macrobenthic assemblages in the vicinity of the cages were recorded a few months following cessation of the farming activities (April'02) (see Chapter 2) (e.g. Marin *et al*., 2007; Vita & Marin, 2007); these observations support the conclusion that seasonal tuna penning practice, together with the often offshore location of tuna farms, allow for mitigation of potentially larger adverse impacts of the activity on benthic habitat (Aksu *et al*., 2010; Moraitis *et al*., 2013; Vezzulli *et al*., 2008; Vita & Marin, 2007). The level and spatial extent of influence of tuna penning on benthic macroinvertebrate assemblages appears to result from accumulation of uneaten feed-fish on the seabed (Holmer *et al*., 2008) (see Chapter 2); hence a controlled feeding regime to reduce feed wastage is another important mitigation measure (Aguado *et al*., 2004), while development of a formulated diet as opposed to feed-fish for use as food for the caged tuna will decrease the impact of the activity on harvest of wild clupeid fish for use as food for the caged tuna (Mourente & Tocher, 2009).

The results from the second study component ([b] above) indicate low (albeit not significant) number and diversity of polychaetes and amphipods, and elevated (albeit not significant) abundance of capitellid polychaetes at the impacted plots over time

(see Chapter 3). Furthermore, a significant high dispersion of samples of the polychaete and amphipod fauna at the impacted plots over time was indicative of stressed macroinvertebrate assemblages, while values of the polychaete/amphipod index showed that the benthic Ecological Quality Status (EQS) changed from 'Poor'/'Moderate' to 'Good' during the study period (Chapter 3). These results were, to some extent, expected (e.g. Aguado-Giménez *et al*., 2015; Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011; Jahani *et al*., 2012; Martinez-Garcia *et al*., 2013, 2018). On the other hand, molluscs and decapods were not sufficiently indicative of ecological change resulting from tuna penning activities (Chapter 3) (but see Charalampos & Drosos, 2008; Putro *et al*., 2017). Taken together, these observatons indicate that, compared to molluscs and decapods, polychaetes and amphipods are better indicators of ecological change of benthic habitat resulting from tuna penning activities, and the latter may therefore be used to decrease the taxonomic effort required in aquaculture monitoring studies, which will allow more extensive and cost-effective monitoring (Olsgard & Sommerfield, 2000).

Results from the third study aspect ([c] above) indicated that the magnitude and spatial extent of influence of tuna ranches on benthic macroinvertebrate assemblages varied significantly spatially; i.e. at the scale of kilometres; and that this depended on the farm size and local marine environmental factors (see Chapter 4). Such findings are not surprising, given that varying levels of influence on benthic habitat are reported for different Mediterranean tuna farms (Aksu *et al*., 2016; Jahani *et al*., 2012; Marin *et al*., 2007; Moraitis *et al*., 2013; Vita & Marin, 2007), which probably result from differences in farm characteristics, and in factors such as water depth and exposure that characterise a given site (e.g. Borja *et al*., 2009c; Tomassetti *et al*., 2009). The northeastern farm included in the present study – the largest of the three studied in terms of holding capacity - showed the highest magnitude of impact, as indicated by the elevated levels of FFBC and 'Bad' EQS classification recorded from the seabed area occupied by the cages (see Chapter 4). This observation corroborates the expectation that benthic ecological quality will be lower at fish farm sites that have a higher total annual production (Borja *et al*., 2009c). On the other hand, the spatial extent of influence appeared largest at one of the two southeastern farms ('Farm 2'), where the influence of tuna penning activities resulted in 'Moderate' EQS at the reference area located some 1 km down-current from the cages, probably due to the

transport of organic matter from uneaten feed-fish present below the tuna cages there via the predominant southern sea currents (see Chapter 4). However, such finding may possibly reflect an 'additive effect' of the two southeastern farms, given that they are located relatively close to each other (1 km apart). This latter observation has important implications for spatial planning of coastal aquaculture activities; while tuna farms have much less adverse influence on the water column, sediment quality, and macrobenthic fauna when located in deep waters characterised by a high energy environment (e.g. Aksu *et al*., 2016; Moraitis *et al*., 2013; Vezzulli *et al*., 2008), farms located relatively close to one another may result in added loading on the environment. The difference in magnitude and spatial extent of the environmental influence of tuna penning on benthic habitat recorded in this study component (see Chapter 4) highlights the importance of including multiple reference areas in environmental monitoring programmes to enable proper assessment of the magnitude and spatial extent of potential impacts of the activity.

The results from the fourth study component ([d] above) showed that the spatial pattern of attributes of the macrobenthic assemblages varied with incremental distance from the tuna cages, and was characterised by a high impact radius on the seabed directly below the cages that was characterised by a significantly high abundance of Capitellidae, a significantly low number and diversity of amphipod families, and low benthic ecological quality ('Bad'/'Poor' and 'Bad'/'Moderate' EQS respectively at the northeastern farm and southeastern 'Farm 1') at both of the investigated tuna farms (see Chapter 5); similar findings are known at fish farms studied by other workers (e.g. Edgar *et al*., 2005; Jahani *et al*., 2012; Karakassis *et al*., 2000; Vita & Marin, 2007). The spatial pattern of stressed benthic assemblages differed between the two farms: 'Poor' ecological quality (e.g. Vita & Marin, 2007) and a significant peak in amphipod diversity (e.g. Karakassis *et al*., 2000; Kutti *et al*., 2007b; Nickell *et al*., 2003) was recorded at a distance of 100 m from the cages of southeastern 'Farm 1', while no peak in diversity was observed at this distance from the northeastern farm, where the ecological quality was 'Good' (see Chapter 5). The latter observation is similar to the findings by Vita and Marin (2007) in their study of the impacts of tuna penning in southeast Spain. Differences in the spatial pattern of benthic biotic diversity at fish farm sites may be attributed to differences in: farm size and operations (Borja *et al*., 2009c), and the hydrodynamic regime (Sanz-Lázaro & Marin, 2011) and sediment

composition and functioning at a given locality (Papageorgiou *et al*., 2010) among the study areas. The spatial pattern of stressed macroinvertebrate assemblages was not evident over the three-year study period considered in this study component. However, 'Good' EQS was recorded within the farm lease areas in 2004, probably due to an improved feed management regime in that period (see Chapter 5). Overall, the importance of temporal replication in the design of environmental impact monitoring studies is highlighted from these findings.

Findings from the fifth study ([e] above) aspect highlighted the importance of using a complementary set of BBIs to obtain an estimate of the EQS as required under the WFD (e.g. Bouchet & Sauriau, 2008; Lavesque *et al*., 2009; Purnomo Putro, 2011), given that different indices give different EQS values, especially for sites having lower ecological quality, and no one index can be taken to be universally ideal (Chapter 6). This conclusion was, to some extent, expected (e.g. Aguado-Giménez *et al*., 2007; Bouchet & Sauriau, 2008; Dauvin *et al*., 2007; Keeley *et al*., 2012; Simboura & Argyrou, 2010; Simonini *et al*., 2009; Spagnolo *et al*., 2014). In general, the BENTIX index tended to overestimate the EQS below the cages compared to the AMBI index, while the M-AMBI index at times indicated lower EQS below the cages compared to the AMBI index. The BOPA and BOPA-FF indices indicated lower ecological quality for the seabed area occupied by the cages compared to the AMBI, BENTIX, and M-AMBI indices. Among the tested BBIs, only the BOPA index showed no significant correlation with sediment physico-chemical attributes, while the M-AMBI index showed the strongest correlation with the fish farm disturbance gradient as indicated by the amphipod data, and only the BOPA and BOPA-FF indices detected the influence of tuna penning activities at a distance of 100 m from the cages (see Chapter 6). Taken together, these observations suggest that the M-AMBI and BOPA-FF indices are more appropriate for obtaining an estimate of the EQS at aquaculture sites in the Maltese Islands compared to the AMBI, BENTIX, and BOPA indices. Workers advise caution in use of the M-AMBI index since the weight it gives to species richness and diversity depends on sample size, habitat type, and season (Simboura & Argyrou, 2010; Subida *et al*., 2012; but see Borja *et al*., 2008). On the other hand, the BOPA-FF index is designed to indicate a specific response to organic enrichment resulting from aquaculture activities (Aguado-Giménez *et al*., 2015), and has the advantage of necessitating only identification of sensitive and first order opportunistic species, thus

decreasing the likelihood of misidentification (Dauvin & Ruellet, 2007) and EG assignment (Dauvin *et al*., 2010) errors; this is particularly advantageous in regions where the ecological strategy of taxa is poorly defined. However, workers advise the use of BBIs only in conjunction with multivariate analyses of physico-chemical and macrobenthic data that has been used traditionally in environmental monitoring of aquaculture (e.g. Aguado-Giménez *et al*., 2015; Blanchet *et al*., 2008; Quintino *et al*., 2012); the underlying reason for this recommendation is that biotic indices based on species tolerance/sensitivity to organic enrichment may not detect changes in macrofaunal assemblages at mild enrichment levels, resulting in information loss (Sampaio *et al*., 2011), while misclassification of the EQS may lead to wrong management decisions with negative consequences on the environment or the fish farmers (Aguado-Giménez *et al*., 2015).

Results from the sixth study aspect ([f] above) showed significantly high POCC in sediment, and 'Bad'/'Poor' EQS classification for the seabed area occupied by the tuna cages during the first years of operation at the northeastern farm; this appeared to result from accumulation of large amounts of uneaten feed-fish on the seabed below the pens (Chapter 7). Benthic ecological quality increased significantly to 'Good'/'High' categorisations during the fallow periods, with no significant difference in values of the BOPA-FF and M-AMBI indices being recorded till the end of the ten-year period considered in the present study (see Chapter 7). These results suggest that the offshore location of the farm and periodic fish farm abatement during winter, together with an improved feed management regime, are sufficient to mitigate the environmental influence of tuna penning activities on benthic habitat and to restrict the disturbance to the 'pulse' type. However, a 'press' disturbance was observed at the southeastern farms towards the end of the study period, when 'Moderate' EQS was recorded for the seabed area occupied by the cages (see Chapter 7). The 'press' disturbance resulting from a cumulative sediment organic loading from the tuna farms possibly ensued due to the higher nutrient loading effecting the coastal waters where the tuna farms are located; i.e. off the southern half of the Maltese Islands (Axiak *et al*., 2000), which are known to support more intense coastal use compared to the northern half of the islands (Mallia *et al*., 2002). Furthermore, the southeastern farms may have had an additive effect, given that they are only 1 km apart (see

above). These latter two observations have important implications for the spatial planning of coastal aquaculture activities, particularly since an offshore 'allocated zone for aquaculture' (AZA) (see Sanchez-Jerez *et al*., 2016) has recently been designated 6 km off the southeastern coast of Malta (Department of Fisheries and Aquaculture-Environment and Resources Authority [DFA-ERA], 2016). Excessive organic loading of the sediment may lead to potential loss of habitat and biodiversity, as well as significantly damage sediment ecological functioning, making fish farming unsustainable in the long term (e.g. Macloed *et al*., 2007; Valdemarsen *et al*., 2015).

## 8.3 Evaluating current environmental management practices and challenges for aquaculture activities

Because of their environmental influence, fish farms are often moved offshore to deeper and more exposed locations (Valdemarsen *et al*., 2015) to enhance the dispersal of dissolved nutrients and prevent water quality impacts (Di Marco *et al*., 2017; Price *et al*., 2015). The dispersal of particulate organic matter from sea cages in deep-water locations is also enhanced due to the longer settling times (Mayor, Zuur, Solan, Paton, & Killham, 2010). Therefore, at such offshore locations, the magnitude of influence of fish farming on benthic habitat is decreased to a level that supports impacted but diverse benthic assemblages, while the large amounts of particulate organic matter that are released from the sea cages are mitigated by the high energy environment and deep waters (Valdemarsen *et al*., 2015). However, frequent objections are raised by fish farmers when their operations are relocated to deep-water sites since they claim an increase in operational risks and costs that are incurred at sites located a considerable distance (i.e. several km) offshore (DFA-ERA, 2016). Workers in the Mediterranean have shown that the influence of fish farms on benthic habitat can be minimised if the activity is sited in appropriate coastal areas; for example, in deep waters (e.g. Aksu *et al*., 2016; Maldonado *et al*., 2005; Moraitis *et al*., 2013; Puhr *et al*., 2017; Vezzulli *et al*., 2008). However, deep-water fish farms do not always have little or no environmental impacts (e.g. Hall-Spencer *et al*., 2006; Lee *et al*., 2006; Valdemarsen *et al*., 2012). The magnitude of influence of fish farms when these are located at sites characterised by deep waters appears to critically depend on the hydrodynamic regime

(Valdermarsen *et al*., 2015). For example, Valdermarsen *et al*. (2015) showed that deep-water fish farms in low current regimes (< 2 cm $s^{-1}$) have a large magnitude of influence on benthic habitat, while ones located in waters that have stronger sea currents (> 3 - 5 cm $s^{-1}$) cause minor changes in sediment quality and in the functioning of macroinvertebrate assemblages.

In the case of multiple, proximate fish farms, an additive effect may lead to significant collective influence on the surrounding marine environment (Fernandes *et al*., 2001), such that strategic environmental management in the form of a joint EIA or Strategic Environmental Impact Assessment (SEIA) is needed (Aguilar-Manjarrez, Soto, & Brummett, 2017). Low to medium levels of impact on ambient nutrient levels in semi-enclosed systems with multiple fish farms are reported (e.g. Maldonado *et al*. 2005; Neofitou & Klaoudatos, 2008; Papageorgiou *et al*., 2010), while fish farming may have far-field effects that extend at low magnitude across regional scales, with potential impacts on the marine ecosystem (Edgar *et al*., 2010). While environmental monitoring programmes for fish farms are designed to identify localised impacts of particulate organic matter in the immediate vicinity of the fish cages, dissolved nutrients that origin from a farm will spread over wider areas in the water column and benthic habitat via invertebrates, and the icthyofauna and other marine vertebrates (Fernandes *et al*., 2001). This may result in little or no influence on benthic trophic levels, or contribute towards increased biomass of primary producers (Price *et al*., 2015). Monitoring of reference sites in areas that do not support fish farms is needed to distinguish region-wide fish farming effects from unrelated, long-term, environmental change (Edgar *et al*., 2010). The selection of reference areas that are distant enough from aquaculture operations and other anthropogenic sources of disturbance, and still reflective of the marine environmental characteristics of the farm lease area, can be a challenge in developed coastal regions (Troell *et al*., 2003). Furthermore, most developed coastal regions are impacted by multiple anthropogenic sources, which are often superimposed on natural variation in marine environmental characteristics (Fernandes *et al*., 2001).

The designation of ‘aquaculture management areas’ (AMAs), which benefit from a collective management system aimed at reducing or eliminating environmental impacts, and social and fish disease risks, may be an important step forward in

achieving sustainable aquaculture practices in AZAs (Aguilar-Manjarrez *et al*., 2017). An entire AZA may be designated as a single AMA depending on the size of the zone and connectivity among farms (Aguilar-Manjarrez *et al*., 2017). AMAs facilitate coordination of environmental and fish health monitoring, as well as facilitating remedial action plans among farms, and allowing for joint management by proximate operators (Aguilar-Manjarrez *et al*., 2017). AMAs also profit farmers by offering an opportunity for coordination of aquaculture licensing, and provision of: joint access to supplies, technical support and markets, certification of produce under an ecosystem approach and conflict resolution with other common resource users (Aguilar-Manjarrez *et al*., 2017).

In the case of tuna farms, the use of feed-fish is suspected of generating a sea surface slime which is derived from fish oil and is pushed inshore by currents (DFA-ERA, 2016), resulting in loss of beach aesthetics and amenity that have a large adverse effect on tourism and recreational use by locals (Katavic, Vicina, & Franicevic, 2003). In the case of Malta, this slime phenomenon is considered to potentially have an adverse influence on the environment, due to its possible negative effects on the sea surface microlayer, on rocky shore habitat (inclusive of the *Cystoseira* WFD bioindicator alga), and on sandy beach habitat found along the eastern coast of the island (DFA-ERA, 2016).

With the rapid increase in global aquaculture production, it is a challenge to find alternative sources of protein and lipids in the feed used for farmed tuna, while the sustainability of using wild bait-fish in the production of fish-feed is questionable (Abdou *et al*., 2017). The use of plant-based proteins and lipids instead of fish oil and fish meal in fish-feed (e.g. Hixson, 2014; Ytrestøyl, Aas, & Åsgård, 2015) decreases the dependence on wild populations of fish for aquaculture feed, and also potentially improves the food conversion ratio, and reduces the environmental impacts of fish farming while increasing economic returns (Abdou *et al*., 2017). Workers have shown that elevated nutrient levels in the water column are restricted to 100 m from the fish cages when commercial manufactored diets are used and over feeding is minimised (Price *et al*., 2015), while fish farms employing manual feeding technique also allow for better control of feed wastage (Tomassetti *et al*., 2016).

The occurrence of wild fish consuming uneaten feed around sea cages and smaller bait fish aggregated at Mediterranean fish farms is well documented (e.g. Sanchez-Jerez *et al*., 2011); the most frequently observed families of such wild fish being Carangidae, Clupeidae, Mugilidae, and Sparidae (Arechavala-Lopez *et al*., 2015). Species of commercial interest such as ABT that aggregate at offshore fish farms become vulnerable to fishing practices within the farm lease area (e.g. Akyol & Ertosluk, 2010; Arechavala-Lopez *et al*., 2015; Bacher, Gordoa, & Sagué, 2012; Sanchez-Jerez *et al*., 2011; Šegvić Bubić *et al*., 2011). In Malta, wild tuna caught by fishermen and which end up with the attached fishing line tangled against the cages or mooring lines, eventually die and are not retrieved; hence end up as carcasses that decompose slowly on the seabed in the vicinity of tuna farms (Arechavala-Lopez *et al*., 2015). Fish remains generated from the harvest of ABT; which amount to 8-10 tonnes per day (Malta Today [MT], 2018) are discarded at sea at a site approved by the local aquaculture authorities (Camilleri, T.C., Aquaculture Director, Department of Fisheries and Aquaculture, MESDC; personal communication, Feburary 13, 2018); and may pose a threat to quality of the water column and benthic habitat in the vicinity of the offal dump site.

While organic loading from fish farms is generally associated with adverse influence on water quality and benthic habitat, reports of enhanced abundance and diversity in the vicinity of fish farms under moderate enrichment levels are also known (Chopin, Cooper, Reid, Cross, & Moore, 2012). In integrated multi-trophic aquaculture (IMTA), inorganic or organic extractive species having a commercial value, for example, algae, and deposit- and suspension- feeding fauna respectively, are used to remove dissolved and particulate organic matter from the water column in intensively farmed aquaculture sites; hence improving water quality and reducing sediment organic loading, while increasing farm productivity (e.g. Neori, Shpigel, Guttman, & Israel, 2017; Price *et al*., 2015; Wartenberg *et al*., 2017). The deposition of organic matter from fish cages to the seabed is not significantly reduced when using suspended-culture systems due to the high settling velocity of fish farm wastes, such that mitigation of benthic influence at IMTA sites may be better achieved using bottom-culture systems (Filgueira, Guyondet, Reid, Grant, & Cranford, 2017).

Overall, the present study has contributed information that may be used to draw cost-effective environmental monitoring programmes and impact mitigation strategies for ABT ranches. In the local context, the present research has provided valuable data for a better understanding of the influence of tuna penning activities on macroinvertebrate assemblages associated with the soft bottom habitat in the Maltese Islands. There is a dearth of local published studies on the influence of tuna farms on benthic habitat (Borg & Schembri, 2001, 2005; Holmer *et al*., 2008), and only a few studies on the influence of tuna penning on macroinvertebrate assemblages deal with collective analyses of monitoring data from multiple tuna farms and reference areas, and replicated sampling times (but see Moraitis *et al*., 2013; Vita & Marin, 2007). More specifically, no previous studies on the effects of tuna penning on benthic habitat have considered before-after impact data, differences in impact among tuna farms, far-field (circa 1-2 km) effects, and long-term patterns of influence; as have been covered in the present study. Furthermore, to the best of the present author's knowledge, there is no study that examined which benthic biological indicators best signal change resulting from tuna penning activities.

## 8.4 Conclusions

In conclusion, the present results showed that tuna penning activities at the northeastern farm resulted in significantly elevated sediment POCC and PONC, and (albeit not significant) abundance of capitellids in the vicinity of the cages, where uneaten feed-fish had accumulated on the seabed. Similar results were obtained 100-200 m away from the cages, hence exceeding the spatial extent of benthic influence reported for Mediterranean sea bream and sea bass farms. The changes in sediment quality and macroinvertebrate assemblages were conspicuous in autumn, towards the end of the tuna penning season, while storms and bottom currents in winter allowed for some recovery in sediment quality and macroinvertebrate assemblages to take place following cessation of the farming activities. The magnitude of influence recorded at the northeastern farm was high compared to that recorded at the two southeastern farms, which corroborated the expectation that benthic ecological quality will be lower at fish farm sites that have a higher total annual production (Borja *et al*., 2009c). On the other hand, the spatial extent of impact appeared largest at one of the southeastern farms ('Farm 2'), where the influence of tuna penning activities extended

some 1-2 km away from the cages, possibly due to the transportation of particulate organic matter there via sea currents. Spatial variation in attributes of the macroinvertebrate assemblages with incremental distance from the cages among tuna farms may be possibly attributed to differences in farm size, operations and management (Borja *et al*., 2009c), in the hydrodynamic regime (Sanz-Lázaro & Marin, 2011), and sediment physico-chemical characteristics and functioning (Papageorgiou *et al*., 2010) of the specific site used for tuna penning. In general, the spatial pattern of stressed macrofaunal assemblages was characterised by a high impact area directly below the cages at the investigated tuna farms, while benthic ecological quality differed between the farms at a distance of 100 - 200 m from the cages; a significant peak in amphipod diversity and 'Poor' ecological quality was observed only at the investigated southeastern farm. Differences among tuna farms was also recorded in the temporal pattern of benthic disturbance during the repeated use of sites for aquaculture; while benthic ecological quality increased from 'Bad' and 'Poor' to 'Good'/'High' categorisations at the northeastern farm during the ten-year study period, a press-type of disturbance was observed at both southeastern farms, where 'Moderate' EQS was recorded at the end of the study period. Such disturbance appeared to result from the cumulative effect of sediment organic loading during several years of tuna penning activities at the farm sites, and may be possibly attributed to an additive effect of the two farms (only 1 km apart), as well as to the higher nutrient loading affecting southern Maltese coastal waters compared to the northern coastal areas of the islands (Axiak *et al*., 2000).

Taken together, these observations indicate that the seasonal nature of tuna penning, together with the often offshore location of tuna farms, allow for mitigation of the potential adverse influence of the activity on benthic habitat, while multiple tuna farms located close to one another may result in added loading on the environment. This conclusion has important implications for the spatial planning of coastal aquaculture activities, given that many countries are moving towards establishing offshore AZAs (Sanchez-Jerez *et al*., 2016). Excessive organic loading of the seabed may significantly damage sediment ecological function, and render fish farming unviable in the long term (Macloed *et al*., 2007; Valdemarsen *et al*., 2015). The importance of reducing feed wastage (Ballester-Moltó *et al*., 2017) and the use of a formulated diet as opposed to whole feed-fish for tuna penning (Aguado *et al*., 2004) to reduce or

eliminate the potential adverse benthic effects of these activities, has already been highlighted by other workers. Given the intensification of aquaculture in off-shore AZAs, measures should be taken to ensure that the potential adverse environmental influence of fish farming activities are eliminated or reduced to an acceptable level such that good benthic quality is maintained in coastal zones. Finally, the high spatio-temporal variation in the magnitude and spatial extent of influence of tuna penning on benthic habitat recorded in the present work highlights the importance of including multiple reference areas, as well as replicated sampling times in aquaculture environmental monitoring studies. BBIs such as BOPA-FF and M-AMBI may be used locally as a complementary set of indices to estimate the EQS at aquaculture sites in order to fulfil the requirements of the WFD in monitoring, while these should also be used in conjunction with multivariate analyses of physico-chemical and macrobenthic data to avoid possible inappropriate management decisions based on erroneous EQS categorisations (Aguado-Giménez *et al*., 2015). In particular, polychaete and amphipod assemblages appear to be good indicators of benthic change resulting from tuna penning activities, and may be used as surrogates for the whole macroinvertebrate assemblage to reduce the taxonomic effort required in aquaculture environmental monitoring studies.

## 8.5 Recommendations for future research

In view of the present findings, a number of recommendations for future research are proposed, as follows:

a) The findings of the present study showed that the pattern of influence of tuna penning activities on benthic habitat in one location cannot be extrapolated to tuna farms in other locations due to differences in characteristics of the farms and of the receiving environment. Additionally, what may be applicable to a Mediterranean farm rearing sea bass and sea bream, may not be applicable to an ABT farm; apart from the difference in the size of the farmed fish, the different type of feed used (i.e. whole feed-fish as opposed to formulated feed) results in varying levels of impact among different Mediterranean fish farms. Significant variation in the influence of tuna penning activities on macrofaunal assemblages among farm locations (km's) indicated that different factors may

influence macroinvertebrate assemblages in the vicinity of fish farms depending on the spatial scale considered. The findings of the present research also showed significant variability in the influence of tuna penning activities on soft bottom habitat along both short-term (i.e. months) and long-term (i.e. years) temporal scales. It is therefore important to avoid generalizations when describing the influence of fish farming activities on the soft bottom habitat, while incorporating both multiple spatial (e.g. Fernandez-Gonzalez *et al*., 2013; Martinez-Garcia *et al*., 2018) and temporal (Fernandes *et al*., 2001) scales in aquaculture environmental monitoring studies is critical, as already highlighted by previous workers. Furthermore, significant variability in the influence of tuna penning activities on benthic habitat at the smallest spatial scale (m's) indicated that at times higher statistical power may be needed for an accurate assessment (Martinez-Garcia *et al*., 2018).

b) The findings of the present research suggested that the influence of tuna penning activities extended up to 1-2 km away from the cages, possibly due to the transportation of particulate organic matter there via sea currents. To truly achieve environmentally sustainable aquaculture practices there is hence need to understand the environmental influence of fish farming activities that goes beyond the immediate vicinity of the farm lease area (Husa *et al*., 2014). Far-field effects are difficult to measure and not routinely monitored (Jansen *et al*., 2016), and there is a gap in knowledge on the spread and persistence of dissolved nutrients from fish farms over larger areas (Price *et al*., 2015). Assessment of far-field effects of fish farm nutrients require different tools than those used in the present research, such as biomarkers and modelling (Skogen, Eknes, Asplin, & Sandvik, 2009). Research on the far-field effects of aquaculture over long temporal scales will help to fully understand the influence of fish farms on the marine ecosystem (Price *et al*., 2015).

c) The present findings indicate that the magnitude and spatial extent of influence of tuna penning activities on macroinvertebrate assemblages may be attributed to the accumulation of uneaten feed-fish on the seabed, as previously suggested by other workers (e.g. Borg & Schembri, 2005; Vita & Marin, 2007; Vita *et al*., 2004a). Since impoverishment of benthic macrofauna decreases the

macrofaunal capacity to degrade organic matter accumulated on the sediment (Valdemarsen *et al*., 2015), it would be useful to establish the threshold at which the amount of uneaten feed-fish on the sediment will significantly impact the benthic macrofauna to maintain environmentally sustainable aquaculture practices. Research on the amount of particulate organic matter produced as a result of poor feed management practices, as well as from fish feeding behavior, has been recommended by other workers (Ballester-Moltó *et al*., 2017). It may be useful to identify the key macroinvertberate taxa that degrade organic matter accumulated on the sediment at fish farm sites; the presence of these taxa could be used to signal adequately functioning macroinvertebrate assemblages at aquaculture sites. In the local context, there is urgent need to gather data on the slime phenomenon observed in the vicinity of tuna farm sites, which is suspected to potentially have an adverse effect on the sea surface microlayer, as well as on the rocky and sandy shore habitats along the coast of Malta when the oily material is pushed inshore by sea currents (DFA-ERA, 2016). The importance of using formulated feed as opposed to whole feed-fish for tuna penning to mitigate potential adverse influence of tuna penning has already been highlighted by other workers (Aguado *et al*., 2004); yet, commercial manufactured diets for cultured tuna species are not available and research on the nutrition of cultured tuna species is still preliminary (see reviews in Buentello, Seoka, & Suarez, 2016; Takeuchi & Haga, 2015), while replacement of fish meal and fish oil in fish-feed with plant-based proteins and lipids to increase the sustainability of the aquaculture industry still faces several biological challenges (Abdou *et al*., 2017). Local research aimed at identifying the wild fish that feed on the uneaten feed deposited on the seabed at tuna farms, will help in understanding the role of scavengers in controlling tuna penning influence on benthic habitats (e.g. Arechavala-Lopez *et al*., 2015; Fernandes *et al*., 2007a; Šegvić Bubić *et al*., 2011; Svane & Barnett, 2008; Vizzini & Mazzola, 2012), while research aimed at assessing the ecological effects of fish remains disposed of at sea from tuna farm sites will further our knowledge of the ecosystem influence of this industry.

d) With regards to the implications for current environmental management practices and challenges for the aquaculture industry, there is a gap in knowledge on the cumulative effects of multiple fish farms in a region (Price *et al*., 2015), while few studies documenting the environmental impact of deep-water fish farms have been carried out to date (e.g. Bannister, Valdemarsen, Hansen, Holmer, & Ervik, 2014; Kutti *et al*., 2007a, b; Valdemarsen *et al*., 2012, 2015). There is an urgent need for gathering data on the risk that concentrated fish farming activities at AZAs may pose in terms of environmental pollution and fish disease (Aguilar-Manjarrez *et al*., 2017); a spatial risk assessment for AZAs should be carried out that would be aimed at understanding the roles of water depth and sea currents, and of the ecosystem to absorb fish farm organic waste, which in turn determine the environmental impacts and fish health risks (Aguilar-Manjarrez *et al*., 2017). Such research should set guidelines on the maximum holding capacity of the farm according to the carrying capacity of the ecosystem, distance between farms, and fish stocking density (Aguilar-Manjarrez *et al*., 2017). Importance should be given to the current regime at the proposed AZA since the level of benthic impact reported below deep-water fish farms appears to be delicately controlled by currents (e.g. Bannister *et al*., 2014; Kutti *et al*., 2007a, b; Valdemarsen *et al*., 2012, 2015). In a more local context, future research should focus on the effectiveness of relocating the tuna farms to the proposed AZAs off the northeastern and southeastern coasts of Malta as an impact mitigation strategy.

e) IMTA systems can be used to reduce the influence of traditional aquaculture systems on water quality and benthic habitat. While the technical validity and economic viability of IMTA has been shown in various successful applications of this new technology (Neori *et al*., 2017), commercialisation of IMTA still faces several challenges (Wartenberg *et al*., 2017). Co-locating the different trophic components is complicated by the influence of water circulation on the dispersal of fish farm wastes (Filgueira *et al*., 2017), while extractive species in offshore aquaculture systems have to withstand the strong currents of open waters (Troell *et al*., 2009). Bottom-culture systems generally exhibit lower survival rates compared to suspension-culture systems as a result of predation and anoxic conditions at the seabed (e.g. Cutajar, 2016; Yokoyama, 2013; Yu,

Hu, Zhou, Li, & Peng, 2012), which may limit the usefulness of IMTA systems in the mitigation of benthic impacts. Identifying the environmental and economic costs of IMTA systems will determine the practical value of using IMTA (Troell *et al*., 2009), depending on acceptable levels of aquaculture impacts, technical feasibility, and economic viability (Ren, Stenton-Dozey, Plew, Fang, & Gall, 2012). The oligotrophic nature of the Mediterranean stimulates suspension-feeding efficiency (Filgueira *et al*., 2017), making IMTA systems ideal for the cultivation of shellfish which do not normally thrive in oligotrophic conditions. In a more local context, no commercial IMTA farms have been developed to date to the best of our knowledge, while preliminary research indicates *Holothuria poli* and *Mytilus galloprovincialis* as potential candidates for IMTA systems in Malta (Cutajar, 2016).

f) Published studies (e.g. Jahani *et al*., 2012; Marin *et al*., 2007; Moraitis *et al*., 2013; Vita & Marin, 2007), and findings from the present study show that tuna penning activities have potentially adverse influence on macroinvertebrate assemblages, depending on the attributes of the farm and of the receiving environment. An understanding of the biology of the key macroinvertebrate taxa associated with the soft bottom habitat will be useful for interpreting differences in macrofaunal assemblages under the influence of fish farms. Results from previous (e.g. Aguado-Giménez *et al*., 2015; Fernandez-Gonzalez *et al*., 2013; Fernandez-Gonzalez & Sanchez-Jerez, 2011; Martinez-Garcia *et al*., 2013, 2018) and present research work show the importance of polychaete and amphipod assemblages as bioindicators of change in soft bottom habitat resulting from the accumulation of fish farm wastes on the sediment; hence, there is need to describe the polychaete and amphipod fauna associated with the soft bottom habitat in Maltese coastal waters (but see Langeneck, Busoni, Aliani, & Castelli, 2017). More detailed study on the influence of tuna penning activities on the ecology of benthic macrofauna is needed; an expanded P-R model that includes simplification of benthic trophic linkages under the influence of increasing fish farm organic loading will increase our understanding of the consequences of environmental degradation resulting from aquaculture activities (Nordström & Bonsdorff, 2017).

g) In relation to the future of environmental monitoring of marine benthic ecosystems under EU directives, while the present research addressed the suitability of BBIs developed under the WFD to measure the EQS at fish farm sites, research aimed at integrating these WFD indices within the assessment of Environmental Quality Status (EnQS) as required by the MSFD is needed (see Borja, Elliott, Carstensen, Heiskanen, & van de Bund, 2010b). Although the two directives overlap, the MSFD requires a holistic ecosystem based approach (Simboura *et al*., 2015) incorporating various qualitative descriptors that cut across habitats and, in combination, produce a single integrative EnQS assessment (Borja *et al*., 2010b). In contrast, WFD indices such as those incorporated in the present research; i.e. infaunal BBIs for soft bottom habitats; are applicable only to particular benthic habitats (Borja *et al*., 2010b). Benthic indicators also need to be defined under the MSFD according to the various types of marine environmental pressures present in the region; hence, different indicators with complementary properties should be used per MSFD descriptor to measure the complex response of the benthic ecosystem (Van Hoey *et al*., 2010). Member state countries need to select common environmental indicators per MSFD descriptor at a regional or sub-regional level; this will be a challenge given the variety in environmental monitoring strategies among nations (Van Hoey *et al*., 2010). A decision tree may then be used to achieve integration at indicator and descriptor levels to produce a holistic EnQS assessment following Borja *et al*. (2010b). Future marine environmental monitoring needs to adopt a stratified sampling strategy to benthic habitat monitoring, and change from a ‘station-oriented’ to a ‘basin-oriented’ sampling strategy to fulfill the requirements of the MSFD (Van Hoey *et al*., 2010).

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# APPENDIX

# CHAPTER 7 – RESULTS OF PAIR-WISE TESTS

**Appendix 1** Results of the *a posteriori* univariate PERMANOVA pair-wise comparisons for the significant 'Im-vs-Co x Ti' interaction term for percent organic carbon content in the sediment recorded from the northeastern farm (NEF) and southeastern 'Farm 2' (SEF 2) (Chapter 7). Level of significance set at 0.05. Im = Impacted plot, Co = Control plots, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| NEF | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Levels | Im-vs-Co |
| Mar'01, Nov'01 | < ** | ns | Nov'01, Jun'07 | > ** | > *** | Jan'03, May'08 | ns | < ** | Mar'04, Jun'07 | > * | > *** | Mar'01 | ns |
| Mar'01, Apr'02 | < * | ns | Nov'01, May'08 | > ** | ns | Jan'03, Apr'09 | ns | < * | Mar'04, May'08 | ns | ns | Nov'01 | > *** |
| Mar'01, Jan'03 | ns | ns | Nov'01, Apr'09 | > * | ns | Apr'03, Nov'03 | ns | ns | Mar'04, Apr'09 | ns | ns | Apr'02 | ns |
| Mar'01, Apr'03 | < ** | < ** | Apr'02, Jan'03 | ns | > * | Apr'03, Mar'04 | ns | ns | Nov'04, Nov'05 | ns | ns | Jan'03 | ns |
| Mar'01, Nov'03 | < ** | < * | Apr'02, Apr'03 | < * | < ** | Apr'03, Nov'04 | > * | ns | Nov'04, Apr'06 | ns | > * | Apr'03 | ns |
| Mar'01, Mar'04 | ns | < * | Apr'02, Nov'03 | ns | ns | Apr'03, Nov'05 | > * | ns | Nov'04, Jun'07 | > ** | > ** | Nov'03 | ns |
| Mar'01, Nov'04 | < ** | < * | Apr'02, Mar'04 | ns | < ** | Apr'03, Apr'06 | ns | > ** | Nov'04, May'08 | ns | ns | Mar'04 | ns |
| Mar'01, Nov'05 | < *** | ns | Apr'02, Nov'04 | ns | < * | Apr'03, Jun'07 | > ** | > *** | Nov'04, Apr'09 | ns | ns | Nov'04 | ns |
| Mar'01, Apr'06 | ns | ns | Apr'02, Nov'05 | ns | ns | Apr'03, May'08 | > * | > * | Nov'05, Apr'06 | ns | ns | Nov'05 | ns |
| Mar'01, Jun'07 | ns | ns | Apr'02, Apr'06 | ns | ns | Apr'03, Apr'09 | > * | ns | Nov'05, Jun'07 | > *** | ns | Apr'06 | ns |
| Mar'01, May'08 | < ** | ns | Apr'02, Jun'07 | > ** | > ** | Nov'03, Mar'04 | ns | ns | Nov'05, May'08 | ns | ns | Jun'07 | < * |
| Mar'01, Apr'09 | < ** | < * | Apr'02, May'08 | ns | ns | Nov'03, Nov'04 | ns | ns | Nov'05, Apr'09 | ns | ns | May'08 | ns |
| Nov'01, Apr'02 | > * | ns | Apr'02, Apr'09 | ns | ns | Nov'03, Nov'05 | ns | ns | Apr'06, Jun'07 | ns | > * | Apr'09 | ns |
| Nov'01, Jan'03 | > * | > *** | Jan'03, Apr'03 | ns | < *** | Nov'03, Apr'06 | ns | ns | Apr'06, May'08 | ns | ns | | |
| Nov'01, Apr'03 | ns | < ** | Jan'03, Nov'03 | ns | < * | Nov'03, Jun'07 | > ** | > ** | Apr'06, Apr'09 | ns | ns | | |
| Nov'01, Nov'03 | > * | ns | Jan'03, Mar'04 | ns | < *** | Nov'03, May'08 | ns | ns | Jun'07, May'08 | < *** | < ** | | |
| Nov'01, Mar'04 | > * | > * | Jan'03, Nov'04 | ns | < ** | Nov'03, Apr'09 | ns | ns | Jun'07, Apr'09 | < ** | < ** | | |
| Nov'01, Nov'04 | > * | ns | Jan'03, Nov'05 | ns | ns | Mar'04, Nov'04 | ns | ns | May'08, Apr'09 | ns | ns | | |
| Nov'01, Nov'05 | > * | ns | Jan'03, Apr'06 | ns | ns | Mar'04, Nov'05 | ns | ns | | | | | |
| Nov'01, Apr'06 | > * | ns | Jan'03, Jun'07 | ns | ns | Mar'04, Apr'06 | ns | > * | | | | | |

**Appendix 1** Continued

| SEF 2 | | | | |
|---|---|---|---|---|
| Groups | Im | Co | | Im-vs-Co |
| Jun'01, Jun'02 | ns | ns | Jun'01 | ns |
| Jun'01, Jun'03 | ns | ns | Jun'02 | ns |
| Jun'01, Jun'04 | ns | ns | Jun'03 | ns |
| Jun'01, Jun'05 | ns | ns | Jun'04 | ns |
| Jun'01, Jun'06 | > *** | > *** | Jun'05 | ns |
| Jun'01, Jun'07 | > ** | > *** | Jun'06 | ns |
| Jun'01, Jun'08 | > ** | > *** | Jun'07 | > *** |
| Jun'01, Jun'09 | ns | ns | Jun'08 | > *** |
| Jun'02, Jun'03 | ns | ns | Jun'09 | ns |
| Jun'02, Jun'04 | ns | ns | | |
| Jun'02, Jun'05 | ns | ns | | |
| Jun'02, Jun'06 | > *** | > ** | | |
| Jun'02, Jun'07 | > * | > ** | | |
| Jun'02, Jun'08 | > * | > ** | | |
| Jun'02, Jun'09 | ns | ns | | |
| Jun'03, Jun'04 | ns | ns | | |
| Jun'03, Jun'05 | ns | ns | | |
| Jun'03, Jun'06 | > ** | > * | | |
| Jun'03, Jun'07 | > * | > ** | | |
| Jun'03, Jun'08 | > ** | > ** | | |
| Jun'03, Jun'09 | ns | ns | | |
| Jun'04, Jun'05 | ns | ns | | |
| Jun'04, Jun'06 | > * | > ** | | |
| Jun'04, Jun'07 | ns | > ** | | |
| Jun'04, Jun'08 | ns | > *** | | |
| Jun'04, Jun'09 | ns | ns | | |
| Jun'05, Jun'06 | ns | > * | | |
| Jun'05, Jun'07 | ns | > * | | |
| Jun'05, Jun'08 | ns | > ** | | |
| Jun'05, Jun'09 | < * | ns | | |
| Jun'06, Jun'07 | ns | ns | | |
| Jun'06, Jun'08 | < * | ns | | |
| Jun'06, Jun'09 | < *** | < * | | |
| Jun'07, Jun'08 | ns | ns | | |
| Jun'07, Jun'09 | < ** | < * | | |
| Jun'08, Jun'09 | < ** | < ** | | |

**Appendix 2** Results of the *a posteriori* univariate PERMANOVA pair-wise comparisons for the significant 'Im-vs-Co x Ti' interaction term for BOPA-FF and M-AMBI indices recorded from the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1), and southeastern 'Farm 2' (SEF 2) (Chapter 7). Level of significance set at 0.05. Im = Impacted plot, Co = Control plots, ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001

| NEF – BOPA-FF | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co |
| Nov'00, Mar'01 | ns | < *** | Mar'01, Apr'06 | ns | ns | Apr'02, Apr'06 | ns | > *** | Nov'03, Mar'04 | > *** | ns |
| Nov'00, Nov'01 | < *** | < ** | Mar'01, Jun'07 | ns | ns | Apr'02, Jun'07 | ns | > ** | Nov'03, Nov'04 | > *** | > ** |
| Nov'00, Apr'02 | ns | < *** | Mar'01, May'08 | ns | < ** | Apr'02, May'08 | ns | ns | Nov'03, Nov'05 | > *** | ns |
| Nov'00, Jan'03 | < * | < *** | Mar'01, Apr'09 | ns | ns | Apr'02, Apr'09 | ns | < ** | Nov'03, Apr'06 | > *** | ns |
| Nov'00, Apr'03 | < ** | < *** | Nov'01, Apr'02 | > *** | < *** | Jan'03, Apr'03 | ns | ns | Nov'03, Jun'07 | > *** | ns |
| Nov'00, Nov'03 | < *** | < ** | Nov'01, Jan'03 | > *** | < * | Jan'03, Nov'03 | < *** | ns | Nov'03, May'08 | > *** | < *** |
| Nov'00, Mar'04 | < * | < *** | Nov'01, Apr'03 | > *** | < * | Jan'03, Mar'04 | ns | > * | Nov'03, Apr'09 | > *** | ns |
| Nov'00, Nov'04 | ns | ns | Nov'01, Nov'03 | ns | ns | Jan'03, Nov'04 | > * | > *** | Mar'04, Nov'04 | ns | > *** |
| Nov'00, Nov'05 | < *** | < *** | Nov'01, Mar'04 | > *** | ns | Jan'03, Nov'05 | ns | ns | Mar'04, Nov'05 | < * | ns |
| Nov'00, Apr'06 | ns | < ** | Nov'01, Nov'04 | > *** | > *** | Jan'03, Apr'06 | ns | ns | Mar'04, Apr'06 | ns | ns |
| Nov'00, Jun'07 | ns | < *** | Nov'01, Nov'05 | > *** | ns | Jan'03, Jun'07 | ns | ns | Mar'04, Jun'07 | ns | ns |
| Nov'00, May'08 | ns | < *** | Nov'01, Apr'06 | > *** | ns | Jan'03, May'08 | > ** | < * | Mar'04, May'08 | > * | < *** |
| Nov'00, Apr'09 | ns | < *** | Nov'01, Jun'07 | > *** | ns | Jan'03, Apr'09 | > * | ns | Mar'04, Apr'09 | ns | < * |
| Mar'01, Nov'01 | < *** | ns | Nov'01, May'08 | > *** | < *** | Apr'03, Nov'03 | < *** | ns | Nov'04, Nov'05 | < *** | < *** |
| Mar'01, Apr'02 | ns | < * | Nov'01, Apr'09 | > *** | < * | Apr'03, Mar'04 | ns | > * | Nov'04, Apr'06 | ns | < *** |
| Mar'01, Jan'03 | ns | ns | Apr'02, Jan'03 | ns | * | Apr'03, Nov'04 | > ** | > *** | Nov'04, Jun'07 | ns | < *** |
| Mar'01, Apr'03 | < * | ns | Apr'02, Apr'03 | < * | ns | Apr'03, Nov'05 | ns | ns | Nov'04, May'08 | ns | < *** |
| Mar'01, Nov'03 | < *** | ns | Apr'02, Nov'03 | < *** | > ** | Apr'03, Apr'06 | ns | > * | Nov'04, Apr'09 | ns | < *** |
| Mar'01, Mar'04 | ns | ns | Apr'02, Mar'04 | ns | > *** | Apr'03, Jun'07 | > * | ns | Nov'05, Apr'06 | < * | ns |
| Mar'01, Nov'04 | ns | > *** | Apr'02, Nov'04 | ns | > *** | Apr'03, May'08 | > *** | < * | Nov'05, Jun'07 | < ** | ns |
| Mar'01, Nov'05 | < ** | ns | Apr'02, Nov'05 | < ** | > *** | Apr'03, Apr'09 | > *** | ns | Nov'05, May'08 | < *** | < *** |

**Appendix 2** Continued

| NEF - BOPA-FF | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co |
| Nov'05, Apr'09 | < *** | ns | Apr'06, May'08 | ns | < *** | Jun'07, May'08 | ns | < ** | May'08, Apr'09 | ns | > ** |
| Apr'06, Jun'07 | ns | ns | Apr'06, Apr'09 | ns | < * | Jun'07, Apr'09 | ns | ns | | | |
| NEF - M-AMBI | | | | | | | | | | | |
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co |
| Nov'00, Mar'01 | < *** | < ** | Mar'01, Nov'04 | > ** | ns | Apr'02, Mar'04 | > * | < * | Apr'03, Nov'05 | > *** | ns |
| Nov'00, Nov'01 | > *** | ns | Mar'01, Nov'05 | > *** | > * | Apr'02, Nov'04 | ns | ns | Apr'03, Apr'06 | ns | < * |
| Nov'00, Apr'02 | ns | < * | Mar'01, Apr'06 | > *** | ns | Apr'02, Nov'05 | > *** | ns | Apr'03, Jun'07 | < ** | < *** |
| Nov'00, Jan'03 | ns | < *** | Mar'01, Jun'07 | ns | < * | Apr'02, Apr'06 | ns | < ** | Apr'03, May'08 | ns | < *** |
| Nov'00, Apr'03 | ns | < ** | Mar'01, May'08 | > * | < * | Apr'02, Jun'07 | < ** | < *** | Apr'03, Apr'09 | ns | ns |
| Nov'00, Nov'03 | > *** | < ** | Mar'01, Apr'09 | > ** | ns | Apr'02, May'08 | < * | < *** | Nov'03, Mar'04 | < *** | ns |
| Nov'00, Mar'04 | ns | < *** | Nov'01, Apr'02 | < *** | < * | Apr'02, Apr'09 | ns | ns | Nov'03, Nov'04 | < *** | ns |
| Nov'00, Nov'04 | ns | ns | Nov'01, Jan'03 | < *** | < *** | Jan'03, Apr'03 | ns | ns | Nov'03, Nov'05 | < ** | ns |
| Nov'00, Nov'05 | > *** | ns | Nov'01, Apr'03 | < *** | < * | Jan'03, Nov'03 | > *** | ns | Nov'03, Apr'06 | < *** | ns |
| Nov'00, Apr'06 | ns | < *** | Nov'01, Nov'03 | ns | < ** | Jan'03, Mar'04 | ns | ns | Nov'03, Jun'07 | < *** | < *** |
| Nov'00, Jun'07 | < ** | < *** | Nov'01, Mar'04 | < ** | < *** | Jan'03, Nov'04 | ns | ns | Nov'03, May'08 | < *** | < *** |
| Nov'00, May'08 | < * | < *** | Nov'01, Nov'04 | < *** | ns | Jan'03, Nov'05 | > *** | > * | Nov'03, Apr'09 | < *** | ns |
| Nov'00, Apr'09 | ns | < *** | Nov'01, Nov'05 | < * | ns | Jan'03, Apr'06 | ns | ns | Mar'04, Nov'04 | < ** | ns |
| Mar'01, Nov'01 | > *** | > ** | Nov'01, Apr'06 | < *** | < *** | Jan'03, Jun'07 | < ** | < ** | Mar'04, Nov'05 | ns | > ** |
| Mar'01, Apr'02 | > *** | ns | Nov'01, Jun'07 | < *** | < *** | Jan'03, May'08 | ns | < ** | Mar'04, Apr'06 | ns | ns |
| Mar'01, Jan'03 | > ** | ns | Nov'01, Apr'09 | < *** | < *** | Jan'03, Apr'09 | ns | ns | Mar'04, Jun'07 | < *** | < *** |
| Mar'01, Apr'03 | > *** | ns | Apr'02, Jan'03 | ns | < * | Apr'03, Nov'03 | > *** | ns | Mar'04, May'08 | < *** | < *** |
| Mar'01, Nov'03 | > *** | ns | Apr'02, Apr'03 | ns | ns | Apr'03, Mar'04 | > * | ns | Mar'04, Apr'09 | < ** | ns |

**Appendix 2** Continued

| NEF - M-AMBI | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co |
| Mar'01, Mar'04 | > *** | ns | Apr'02, Nov'03 | > *** | ns | Apr'03, Nov'04 | ns | ns | Nov'04, Nov'05 | > *** | ns |
| Nov'04, Apr'06 | ns | < * | Nov'05, Apr'06 | < *** | < ** | Apr'06, Jun'07 | < ** | < ** | Jun'07, Apr'09 | ns | > *** |
| Nov'04, Jun'07 | ns | < *** | Nov'05, Jun'07 | < *** | < *** | Apr'06, May'08 | < * | < ** | May'08, Apr'09 | ns | > *** |
| Nov'04, May'08 | ns | < *** | Nov'05, May'08 | < *** | < *** | Apr'06, Apr'09 | ns | ns | | | |
| Nov'04, Apr'09 | ns | ns | Nov'05, Apr'09 | < *** | < * | Jun'07, May'08 | ns | ns | | | |

| Im-vs-Co | | |
|---|---|---|
| Level | BOPA-FF | M-AMBI |
| Nov'00 | ns | < * |
| Mar'01 | ns | ns |
| Nov'01 | > *** | < *** |
| Apr'02 | < ** | < *** |
| Jan'03 | ns | < *** |
| Apr'03 | ns | < *** |
| Nov'03 | > *** | < *** |
| Mar'04 | ns | < *** |
| Nov'04 | ns | < * |
| Nov'05 | > *** | < *** |
| Apr'06 | ns | < *** |
| Jun'07 | ns | < *** |
| May'08 | < *** | < *** |
| Apr'09 | < * | < *** |

**Appendix 2** Continued

| | SEF 1 | | | | | | |
|---|---|---|---|---|---|---|---|
| | BOPA-FF | | MAMBI | | SEF 1 | Im-vs-Co | |
| Groups | Im | Co | Im | Co | Level | BOPA-FF | M-AMBI |
| Oct'02, Oct'03 | < ** | < * | > * | ns | Oct'02 | ns | < * |
| Oct'02, Oct'04 | ns | ns | ns | ns | Oct'03 | > ** | < ** |
| Oct'02, Oct'05 | < *** | ns | > *** | ns | Oct'04 | ns | ns |
| Oct'02, Jun'06 | ns | < ** | > *** | > * | Oct'05 | > *** | < *** |
| Oct'02, Jun'07 | ns | ns | ns | ns | Jun'06 | ns | > *** |
| Oct'02, Jun'08 | < ** | ns | ns | ns | Jun'07 | ns | < * |
| Oct'02, Jun'09 | < ** | < *** | ns | ns | Jun'08 | > *** | < ** |
| Oct'03, Oct'04 | > ** | ns | ns | ns | Jun'09 | ns | ns |
| Oct'03, Oct'05 | < ** | ns | > ** | ns | | | |
| Oct'03, Jun'06 | ns | ns | ns | ns | | | |
| Oct'03, Jun'07 | > * | > ** | < * | < * | | | |
| Oct'03, Jun'08 | ns | ns | ns | < ** | | | |
| Oct'03, Jun'09 | ns | ns | ns | ns | | | |
| Oct'04, Oct'05 | < *** | ns | > *** | ns | | | |
| Oct'04, Jun'06 | ns | < * | > ** | ns | | | |
| Oct'04, Jun'07 | ns | ns | ns | < * | | | |
| Oct'04, Jun'08 | < * | ns | ns | < ** | | | |
| Oct'04, Jun'09 | < ** | < ** | ns | ns | | | |
| Oct'05, Jun'06 | > *** | ns | < * | ns | | | |
| Oct'05, Jun'07 | > *** | ns | < *** | < * | | | |
| Oct'05, Jun'08 | > *** | ns | < *** | < *** | | | |
| Oct'05, Jun'09 | > *** | < * | < ** | ns | | | |
| Jun'06, Jun'07 | ns | > ** | < *** | < ** | | | |
| Jun'06, Jun'08 | ns | > * | < *** | < *** | | | |
| Jun'06, Jun'09 | ns | ns | < * | ns | | | |
| Jun'07, Jun'08 | < * | ns | ns | ns | | | |
| Jun'07, Jun'09 | < ** | < *** | ns | ns | | | |
| Jun'08, Jun'09 | ns | < ** | ns | ns | | | |

**Appendix 2** Continued

| SEF 2 | | | | | | | |
|---|---|---|---|---|---|---|---|
| | BOPA-FF | | M-AMBI | | | Im-vs-Co | |
| Groups | Im | Co | Im | Co | | BOPA-FF | M-AMBI |
| Jun'01, Jun'02 | < * | > *** | ns | > ** | Jun'01 | ns | ns |
| Jun'01, Jun'03 | > *** | > *** | ns | ns | Jun'02 | > *** | ns |
| Jun'01, Jun'04 | > *** | > *** | > *** | ns | Jun'03 | ns | < * |
| Jun'01, Jun'05 | > *** | > *** | > *** | ns | Jun'04 | ns | < *** |
| Jun'01, Jun'06 | > * | < * | > *** | > *** | Jun'05 | ns | < *** |
| Jun'01, Jun'07 | ns | ns | ns | ns | Jun'06 | < *** | < *** |
| Jun'01, Jun'08 | < *** | ns | > *** | ns | Jun'07 | ns | < ** |
| Jun'01, Jun'09 | < *** | < ** | > *** | ns | Jun'08 | > *** | < *** |
| Jun'02, Jun'03 | > *** | ns | ns | < *** | Jun'09 | > * | < *** |
| Jun'02, Jun'04 | > *** | ns | > * | ns | | | |
| Jun'02, Jun'05 | > *** | ns | > *** | < ** | | | |
| Jun'02, Jun'06 | > ** | < *** | > *** | ns | | | |
| Jun'02, Jun'07 | ns | < *** | ns | < *** | | | |
| Jun'02, Jun'08 | ns | < *** | > ** | < *** | | | |
| Jun'02, Jun'09 | ns | < *** | > *** | ns | | | |
| Jun'03, Jun'04 | ns | ns | > * | > * | | | |
| Jun'03, Jun'05 | ns | ns | > *** | ns | | | |
| Jun'03, Jun'06 | < * | < *** | > *** | > *** | | | |
| Jun'03, Jun'07 | < ** | < *** | ns | ns | | | |
| Jun'03, Jun'08 | < *** | < *** | > ** | ns | | | |
| Jun'03, Jun'09 | < *** | < *** | > *** | ns | | | |
| Jun'04, Jun'05 | ns | ns | ns | ns | | | |
| Jun'04, Jun'06 | > * | < *** | > * | > * | | | |
| Jun'04, Jun'07 | > *** | < *** | ns | < * | | | |
| Jun'04, Jun'08 | > *** | < *** | ns | < *** | | | |
| Jun'04, Jun'09 | > *** | < *** | ns | ns | | | |
| Jun'05, Jun'06 | < * | < *** | ns | > ** | | | |
| Jun'05, Jun'07 | < *** | < *** | < * | ns | | | |
| Jun'05, Jun'08 | < *** | < *** | ns | < ** | | | |
| Jun'05, Jun'09 | < *** | < *** | ns | ns | | | |
| Jun'06, Jun'07 | < * | > *** | < ** | < *** | | | |
| Jun'06, Jun'08 | < *** | > ** | ns | < *** | | | |
| Jun'06, Jun'09 | < *** | ns | ns | ns | | | |
| Jun'07, Jun'08 | ns | ns | ns | ns | | | |
| Jun'07, Jun'09 | < ** | < *** | > ** | ns | | | |
| Jun'08, Jun'09 | ns | < ** | ns | > * | | | |

**Appendix 3** Results of the *a posteriori* multivariate PERMDISP pair-wise comparisons for the significant 'Im-vs-Co x Ti' interaction term for sediment physico-chemical data recorded from the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1) and southeastern 'Farm 2' (SEF 2) (Chapter 7). Variables included in the analyses are normalised values of mean sediment grain size (phi), and percent organic carbon content and percent organic nitrogen content of the sediment (Chapter 7). Level of significance set at 0.05. Im= Impacted plot, Co = Control plots, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| NEF | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Level | Im-vs-Co |
| Mar'01, Nov'01 | * | ns | Nov'01, Jun'07 | * | ns | Jan'03, May'08 | ns | ns | Mar'04, Jun'07 | ns | * | Nov'00 | ** |
| Mar'01, Apr'02 | ns | ns | Nov'01, May'08 | * | ns | Jan'03, Apr'09 | ns | ** | Mar'04, May'08 | * | * | Mar'01 | ** |
| Mar'01, Jan'03 | ns | *** | Nov'01, Apr'09 | * | ns | Apr'03, Nov'03 | ns | ns | Mar'04, Apr'09 | ns | ns | Nov'01 | ** |
| Mar'01, Apr'03 | * | ns | Apr'02, Jan'03 | ns | * | Apr'03, Mar'04 | ns | ns | Nov'04, Nov'05 | ns | ns | Apr'02 | ns |
| Mar'01, Nov'03 | ns | ns | Apr'02, Apr'03 | ns | ns | Apr'03, Nov'04 | ns | ** | Nov'04, Apr'06 | ns | ** | Jan'03 | ns |
| Mar'01, Mar'04 | * | ns | Apr'02, Nov'03 | ns | ns | Apr'03, Nov'05 | ns | ns | Nov'04, Jun'07 | ns | *** | Apr'03 | * |
| Mar'01, Nov'04 | * | *** | Apr'02, Mar'04 | ns | ns | Apr'03, Apr'06 | ns | ns | Nov'04, May'08 | * | *** | Nov'03 | ns |
| Mar'01, Nov'05 | * | ns | Apr'02, Nov'04 | ns | *** | Apr'03, Jun'07 | ns | ns | Nov'04, Apr'09 | ns | ns | Mar'04 | ns |
| Mar'01, Apr'06 | * | ns | Apr'02, Nov'05 | ns | ns | Apr'03, May'08 | * | ns | Nov'05, Apr'06 | ns | ns | Nov'04 | ns |
| Mar'01, Jun'07 | ns | ns | Apr'02, Apr'06 | ns | ns | Apr'03, Apr'09 | ns | ns | Nov'05, Jun'07 | ns | ns | Nov'05 | ns |
| Mar'01, May'08 | ns | ns | Apr'02, Jun'07 | ns | ns | Nov'03, Mar'04 | ns | ns | Nov'05, May'08 | * | * | Apr'06 | ns |
| Mar'01, Apr'09 | * | ns | Apr'02, May'08 | ns | ns | Nov'03, Nov'04 | ns | ns | Nov'05, Apr'09 | ns | ns | Jun'07 | ns |
| Nov'01, Apr'02 | * | ns | Apr'02, Apr'09 | ns | ns | Nov'03, Nov'05 | ns | ns | Apr'06, Jun'07 | ns | ns | May'08 | ns |
| Nov'01, Jan'03 | * | * | Jan'03, Apr'03 | ns | ** | Nov'03, Apr'06 | ns | ns | Apr'06, May'08 | * | ns | Apr'09 | ns |
| Nov'01, Apr'03 | ns | ns | Jan'03, Nov'03 | ns | ** | Nov'03, Jun'07 | ns | ns | Apr'06, Apr'09 | ns | ns | | |
| Nov'01, Nov'03 | * | ns | Jan'03, Mar'04 | ns | *** | Nov'03, May'08 | ns | ns | Jun'07, May'08 | ns | ns | | |
| Nov'01, Mar'04 | ns | ns | Jan'03, Nov'04 | ns | *** | Nov'03, Apr'09 | ns | ns | Jun'07, Apr'09 | ns | ** | | |
| Nov'01, Nov'04 | ns | ** | Jan'03, Nov'05 | ns | ** | Mar'04, Nov'04 | ns | * | May'08, Apr'09 | * | ** | | |
| Nov'01, Nov'05 | ns | ns | Jan'03, Apr'06 | ns | ** | Mar'04, Nov'05 | ns | ns | | | | | |
| Nov'01, Apr'06 | ns | ns | Jan'03, Jun'07 | ns | * | Mar'04, Apr'06 | ns | ns | | | | | |

**Appendix 3** Continued

| SEF 1 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Level | Im-vs-Co |
| Oct'02, Oct'03 | ns | * | Oct'03, Oct'04 | ns | ns | Oct'04, Jun'06 | ns | ns | Oct'05, Jun'09 | ns | * | Oct'02 | ** |
| Oct'02, Oct'04 | * | ns | Oct'03, Oct'05 | ns | ns | Oct'04, Jun'07 | ns | ns | Jun'06, Jun'07 | ns | ns | Oct'03 | * |
| Oct'02, Oct'05 | * | ns | Oct'03, Jun'06 | ns | ns | Oct'04, Jun'08 | ns | ns | Jun'06, Jun'08 | ns | ns | Oct'04 | ns |
| Oct'02, Jun'06 | * | ns | Oct'03, Jun'07 | ns | ns | Oct'04, Jun'09 | ns | * | Jun'06, Jun'09 | ns | * | Oct'05 | ns |
| Oct'02, Jun'07 | * | ns | Oct'03, Jun'08 | ns | ns | Oct'05, Jun'06 | ns | ns | Jun'07, Jun'08 | ns | ns | Jun'06 | ns |
| Oct'02, Jun'08 | * | ns | Oct'03, Jun'09 | ns | * | Oct'05, Jun'07 | ns | ns | Jun'07, Jun'09 | ns | ns | Jun'07 | ns |
| Oct'02, Jun'09 | ns | ** | Oct'04, Oct'05 | ns | ns | Oct'05, Jun'08 | ns | ns | Jun'08, Jun'09 | ns | ns | Jun'08 | ns |
| | | | | | | | | | | | | Jun'09 | ns |

| SEF 2 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Level | Im-vs-Co |
| Jun'01, Jun'02 | ns | ns | Jun'02, Jun'07 | ** | ** | Jun'04, Jun'08 | ns | *** | Jun'01 | ns |
| Jun'01, Jun'03 | ns | ** | Jun'02, Jun'08 | ** | ** | Jun'04, Jun'09 | ns | ns | Jun'02 | ns |
| Jun'01, Jun'04 | ns | ns | Jun'02, Jun'09 | * | ns | Jun'05, Jun'06 | ns | *** | Jun'03 | ns |
| Jun'01, Jun'05 | ns | * | Jun'03, Jun'04 | ns | * | Jun'05, Jun'07 | ns | *** | Jun'04 | ns |
| Jun'01, Jun'06 | ns | ns | Jun'03, Jun'05 | ns | ns | Jun'05, Jun'08 | ns | *** | Jun'05 | ns |
| Jun'01, Jun'07 | ns | * | Jun'03, Jun'06 | ns | *** | Jun'05, Jun'09 | ns | ns | Jun'06 | ns |
| Jun'01, Jun'08 | ns | * | Jun'03, Jun'07 | ns | *** | Jun'06, Jun'07 | ns | ns | Jun'07 | ns |
| Jun'01, Jun'09 | ns | ns | Jun'03, Jun'08 | ns | **** | Jun'06, Jun'08 | ns | ns | Jun'08 | ns |
| Jun'02, Jun'03 | ns | ns | Jun'03, Jun'09 | ns | ns | Jun'06, Jun'09 | * | * | Jun'09 | ns |
| Jun'02, Jun'04 | ns | ns | Jun'04, Jun'05 | ns | ns | Jun'07, Jun'08 | ns | ns | | |
| Jun'02, Jun'05 | ns | ns | Jun'04, Jun'06 | ns | ** | Jun'07, Jun'09 | *** | * | | |
| Jun02, Jun'06 | ns | ** | Jun'04, Jun'07 | ns | ** | Jun'08, Jun'09 | *** | * | | |

**Appendix 4** Results of the *a posteriori* multivariate PERMANOVA and PERMDISP pair-wise comparisons for the significant 'Im-vs-Co x Ti' interaction term for polychaete and amphipod assemblages recorded from the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1) and southeastern 'Farm 2' (SEF 2) (Chapter 7). Variables included in the analysis are fourth-root transformed family abundance data (Chapter 7). Level of significant set at 0.05. Im= Impacted plot, Co = Control plots, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

NEF – Polychaete family abundance - PERMANOVA

| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nov'00, Mar'01 | *** | *** | Mar'01, Apr'03 | *** | *** | Apr'02, Nov'04 | *** | *** | Apr'03, Jun'07 | *** | *** | Nov'04, Apr'09 | *** | *** |
| Nov'00, Nov'01 | *** | *** | Mar'01, Nov'03 | *** | *** | Apr'02, Nov'05 | ** | *** | Apr'03, May'08 | *** | *** | Nov'05, Apr'06 | * | *** |
| Nov'00, Apr'02 | *** | *** | Mar'01, Mar'04 | *** | *** | Apr'02, Apr'06 | ** | *** | Apr'03, Apr'09 | *** | *** | Nov'05, Jun'07 | *** | *** |
| Nov'00, Jan'03 | *** | *** | Mar'01, Nov'04 | ** | *** | Apr'02, Jun'07 | *** | *** | Nov'03, Mar'04 | *** | ** | Nov'05, May'08 | *** | *** |
| Nov'00, Apr'03 | *** | *** | Mar'01, Apr'09 | *** | *** | Apr'02, May'08 | *** | *** | Nov'03, Nov'04 | *** | *** | Nov'05, Apr'09 | *** | *** |
| Nov'00, Nov'03 | *** | *** | Nov'01, Apr'02 | *** | *** | Apr'02, Apr'09 | *** | *** | Nov'03, Nov'05 | *** | * | Apr'06, Jun'07 | * | *** |
| Nov'00, Mar'04 | *** | *** | Nov'01, Jan'03 | *** | *** | Jan'03, Apr'03 | *** | *** | Nov'03, Apr'06 | *** | *** | Apr'06, May'08 | ns | *** |
| Nov'00, Nov'04 | ** | *** | Nov'01, Apr'03 | *** | *** | Jan'03, Nov'03 | *** | * | Nov'03, Jun'07 | *** | *** | Apr'06, Apr'09 | * | *** |
| Nov'00, Nov'05 | *** | *** | Nov'01, Nov'03 | *** | *** | Jan'03, Nov'04 | *** | *** | Nov'03, May'08 | *** | *** | Jun'07, May'08 | ** | *** |
| Nov'00, Apr'06 | *** | *** | Nov'01, Mar'04 | *** | *** | Jan'03, Mar'04 | *** | *** | Nov'03, Apr'09 | *** | *** | Jun'07, Apr'09 | *** | *** |
| Nov'00, Jun'07 | *** | *** | Nov'01, Nov'04 | *** | *** | Jan'03, Nov'05 | ** | ** | Mar'04, Nov'04 | *** | *** | May'08, Apr'09 | ** | *** |
| Nov'00, May'08 | *** | *** | Nov'01, Nov'05 | *** | *** | Jan'03, Apr'06 | *** | *** | Mar'04, Nov'05 | ns | *** | Level | Im-vs-Co | Level | Im-vs-Co |
| Nov'00, Apr'09 | *** | *** | Nov'01, Apr'06 | *** | *** | Jan'03, Jun'07 | *** | *** | Mar'04, Apr'06 | *** | *** | Nov'00 | ns | Nov'04 | ns |
| Mar'01, Nov'01 | *** | *** | Nov'01, Jun'07 | *** | *** | Jan'03, May'08 | *** | *** | Mar'04, Jun'07 | *** | *** | Mar'01 | ns | Nov'05 | *** |
| Mar'01, Apr'02 | *** | *** | Nov'01, May'08 | *** | *** | Jan'03, Apr'09 | *** | *** | Mar'04, May'08 | *** | *** | Nov'01 | *** | Apr'06 | *** |
| Mar'01, Jan'03 | *** | *** | Nov'01, Apr'09 | *** | *** | Apr'03, Nov'03 | *** | *** | Mar'04, Apr'09 | *** | *** | Apr'02 | *** | Jun'07 | *** |
| Mar'01, Nov'05 | *** | *** | Apr'02, Jan'03 | *** | *** | Apr'03, Mar'04 | *** | *** | Nov'04, Nov'05 | *** | *** | Jan'03 | *** | May'08 | *** |
| Mar'01, Apr'06 | *** | *** | Apr'02, Apr'03 | *** | *** | Apr'03, Nov'04 | *** | *** | Nov'04, Apr'06 | *** | *** | Apr'03 | *** | Apr'09 | *** |
| Mar'01, Jun'07 | *** | *** | Apr'02, Nov'03 | *** | *** | Apr'03, Nov'05 | *** | *** | Nov'04, Jun'07 | *** | *** | Nov'03 | *** | | |
| Mar'01, May'08 | *** | *** | Apr'02, Mar'04 | ** | *** | Apr'03, Apr'06 | *** | *** | Nov'04, May'08 | *** | *** | Mar'04 | *** | | |

**Appendix 4** Continued

| NEF – Polychaete family abundance - PERMDISP | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Level | Im-vs-Co |
| Nov'00, Mar'01 | * | *** | Mar'01, May'08 | ns | ns | Jan'03, Apr'03 | ns | ns | Nov'03, Apr'09 | ns | ** | Nov'00 | ns |
| Nov'00, Nov'01 | *** | ns | Mar'01, Apr'09 | * | ns | Jan'03, Nov'03 | ns | ns | Mar'04, Nov'04 | ns | ** | Mar'01 | ** |
| Nov'00, Apr'02 | * | *** | Nov'01, Apr'02 | ** | *** | Jan'03, Mar'04 | ** | * | Mar'04, Nov'05 | ns | *** | Nov'01 | ns |
| Nov'00, Jan'03 | *** | ** | Nov'01, Jan'03 | ns | ns | Jan'03, Nov'04 | ** | ns | Mar'04, Apr'06 | ns | ns | Apr'02 | *** |
| Nov'00, Apr'03 | *** | ** | Nov'01, Apr'03 | ns | ns | Jan'03, Nov'05 | *** | * | Mar'04, Jun'07 | * | ns | Jan'03 | ns |
| Nov'00, Nov'03 | *** | ns | Nov'01, Nov'03 | ns | ns | Jan'03, Apr'06 | * | ns | Mar'04, May'08 | ns | ns | Apr'03 | ns |
| Nov'00, Mar'04 | ns | *** | Nov'01, Mar'04 | *** | ** | Jan'03, Jun'07 | ns | *** | Mar'04, Apr'09 | *** | ns | Nov'03 | ns |
| Nov'00, Nov'04 | ns | ns | Nov'01, Nov'04 | *** | ns | Jan'03, May'08 | * | *** | Nov'04, Nov'05 | ns | ns | Mar'04 | *** |
| Nov'00, Nov'05 | ns | ns | Nov'01, Nov'05 | *** | ns | Jan'03, Apr'09 | ns | * | Nov'04, Apr'06 | ns | ** | Nov'04 | ns |
| Nov'00, Apr'06 | ns | *** | Nov'01, Apr'06 | ** | * | Apr'03, Nov'03 | ns | ns | Nov'04, Jun'07 | ** | *** | Nov'05 | ** |
| Nov'00, Jun'07 | *** | *** | Nov'01, Jun'07 | * | *** | Apr'03, Mar'04 | *** | ns | Nov'04, May'08 | ns | *** | Apr'06 | *** |
| Nov'00, May'08 | * | *** | Nov'01, May'08 | ** | *** | Apr'03, Nov'04 | *** | * | Nov'04, Apr'09 | *** | ** | Jun'07 | *** |
| Nov'00, Apr'09 | *** | *** | Nov'01, Apr'09 | ns | ** | Apr'03, Nov'05 | *** | *** | Nov'05, Apr'06 | ns | *** | May'08 | *** |
| Mar'01, Nov'01 | ns | ** | Apr'02, Jan'03 | * | ** | Apr'03, Apr'06 | ** | ns | Nov'05, Jun'07 | ** | *** | Apr'09 | ns |
| Mar'01, Apr'02 | ns | ns | Apr'02, Apr'03 | ** | * | Apr'03, Jun'07 | ** | ** | Nov'05, May'08 | * | *** | | |
| Mar'01, Jan'03 | ns | * | Apr'02, Nov'03 | * | *** | Apr'03, May'08 | *** | ** | Nov'05, Apr'09 | *** | *** | | |
| Mar'01, Apr'03 | * | ns | Apr'02, Mar'04 | ns | ns | Apr'03, Apr'09 | ns | ns | Apr'06, Jun'07 | ns | * | | |
| Mar'01, Nov'03 | ns | ** | Apr'02, Nov'04 | ns | *** | Nov'03, Mar'04 | ** | ** | Apr'06, May'08 | ns | * | | |
| Mar'01, Mar'04 | ns | ns | Apr'02, Nov'05 | * | *** | Nov'03, Nov'04 | ** | ns | Apr'06, Apr'09 | *** | ns | | |
| Mar'01, Nov'04 | ns | ** | Apr'02, Apr'06 | ns | ns | Nov'03, Nov'05 | *** | ns | Jun'07, May'08 | ns | ns | | |
| Mar'01, Nov'05 | * | *** | Apr'02, Jun'07 | ns | ns | Nov'03, Apr'06 | * | * | Jun'07, Apr'09 | ** | * | | |
| Mar'01, Apr'06 | ns | ns | Apr'02, May'08 | ns | ns | Nov'03, Jun'07 | ns | *** | May'08, Apr'09 | *** | * | | |
| Mar'01, Jun'07 | ns | * | Apr'02, Apr'09 | *** | ns | Nov'03, May'08 | * | *** | | | | | |

**Appendix 4** Continued

| NEF – Amphipod family abundance - PERMANOVA | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Level | Im-vs-Co |
| Nov'00, Mar'01 | ** | *** | Mar'01, May'08 | *** | *** | Jan'03, Apr'03 | *** | *** | Nov'03, Apr'09 | *** | *** | Nov'00 | ns |
| Nov'00, Nov'01 | *** | *** | Mar'01, Apr'09 | *** | *** | Jan'03, Nov'03 | *** | * | Mar'04, Nov'04 | *** | *** | Mar'01 | ns |
| Nov'00, Apr'02 | *** | *** | Nov'01, Apr'02 | *** | *** | Jan'03, Mar'04 | *** | *** | Mar'04, Nov'05 | * | *** | Nov'01 | *** |
| Nov'00, Jan'03 | *** | *** | Nov'01, Jan'03 | *** | * | Jan'03, Nov'04 | *** | *** | Mar'04, Apr'06 | ns | *** | Apr'02 | *** |
| Nov'00, Apr'03 | *** | *** | Nov'01, Apr'03 | *** | *** | Jan'03, Nov'05 | *** | * | Mar'04, Jun'07 | ** | *** | Jan'03 | *** |
| Nov'00, Nov'03 | *** | *** | Nov'01, Nov'03 | ns | ns | Jan'03, Apr'06 | ** | *** | Mar'04, May'08 | *** | *** | Apr'03 | *** |
| Nov'00, Mar'04 | *** | *** | Nov'01, Mar'04 | *** | ** | Jan'03, Jun'07 | *** | *** | Mar'04, Apr'09 | ** | *** | Nov'03 | *** |
| Nov'00, Nov'04 | ns | * | Nov'01, Nov'04 | *** | *** | Jan'03, May'08 | *** | *** | Nov'04, Nov'05 | *** | *** | Mar'04 | *** |
| Nov'00, Nov'05 | *** | *** | Nov'01, Nov'05 | *** | ns | Jan'03, Apr'09 | *** | *** | Nov'04, Apr'06 | *** | *** | Nov'04 | ns |
| Nov'00, Apr'06 | *** | *** | Nov'01, Apr'06 | *** | *** | Apr'03, Nov'03 | *** | *** | Nov'04, Jun'07 | *** | *** | Nov'05 | *** |
| Nov'00, Jun'07 | *** | *** | Nov'01, Jun'07 | *** | *** | Apr'03, Mar'04 | *** | *** | Nov'04, May'08 | *** | *** | Apr'06 | *** |
| Nov'00, May'08 | *** | *** | Nov'01, May'08 | *** | *** | Apr'03, Nov'04 | *** | *** | Nov'04, Apr'09 | *** | *** | Jun'07 | *** |
| Nov'00, Apr'09 | *** | *** | Nov'01, Apr'09 | *** | *** | Apr'03, Nov'05 | *** | *** | Nov'05, Apr'06 | ** | *** | May'08 | *** |
| Mar'01, Nov'01 | *** | *** | Apr'02, Jan'03 | ** | ** | Apr'03, Apr'06 | ** | *** | Nov'05, Jun'07 | ** | *** | Apr'09 | *** |
| Mar'01, Apr'02 | *** | *** | Apr'02, Apr'03 | *** | *** | Apr'03, Jun'07 | *** | *** | Nov'05, May'08 | *** | *** | | |
| Mar'01, Jan'03 | *** | *** | Apr'02, Nov'03 | *** | *** | Apr'03, May'08 | *** | *** | Nov'05, Apr'09 | *** | *** | | |
| Mar'01, Apr'03 | *** | *** | Apr'02, Mar'04 | ** | *** | Apr'03, Apr'09 | *** | *** | Apr'06, Jun'07 | * | *** | | |
| Mar'01, Nov'03 | *** | *** | Apr'02, Nov'04 | *** | *** | Nov'03, Mar'04 | *** | *** | Apr'06, May'08 | *** | *** | | |
| Mar'01, Mar'04 | ** | *** | Apr'02, Nov'05 | *** | *** | Nov'03, Nov'04 | *** | *** | Apr'06, Apr'09 | ns | *** | | |
| Mar'01, Nov'04 | * | *** | Apr'02, Apr'06 | *** | *** | Nov'03, Nov'05 | ns | ns | Jun'07, May'08 | *** | *** | | |
| Mar'01, Nov'05 | *** | *** | Apr'02, Jun'07 | *** | *** | Nov'03, Apr'06 | *** | *** | Jun'07, Apr'09 | ** | *** | | |
| Mar'01, Apr'06 | ** | *** | Apr'02, May'08 | *** | *** | Nov'03, Jun'07 | *** | *** | May'08, Apr'09 | *** | *** | | |
| Mar'01, Jun'07 | *** | *** | Apr'02, Apr'09 | *** | *** | Nov'03, May'08 | *** | *** | | | | | |

**Appendix 4** Continued

| NEF – Amphipod family abundance - PERMDISP | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Groups | Im | Co | Level | Im-vs-Co |
| Nov'00, Mar'01 | ns | ns | Mar'01, May'08 | ns | ns | Jan'03, Apr'03 | ns | ns | Nov'03, Apr'09 | *** | ns | Nov'00 | ns |
| Nov'00, Nov'01 | *** | ns | Mar'01, Apr'09 | ns | ns | Jan'03, Nov'03 | *** | ns | Mar'04, Nov'04 | ns | ns | Mar'01 | ns |
| Nov'00, Apr'02 | * | ns | Nov'01, Apr'02 | *** | ns | Jan'03, Mar'04 | ns | * | Mar'04, Nov'05 | * | *** | Nov'01 | *** |
| Nov'00, Jan'03 | ns | ns | Nov'01, Jan'03 | *** | ns | Jan'03, Nov'04 | ns | ns | Mar'04, Apr'06 | ns | ns | Apr'02 | ns |
| Nov'00, Apr'03 | ns | ns | Nov'01, Apr'03 | *** | ns | Jan'03, Nov'05 | ** | ns | Mar'04, Jun'07 | * | ns | Jan'03 | ns |
| Nov'00, Nov'03 | *** | ns | Nov'01, Nov'03 | ns | ns | Jan'03, Apr'06 | ns | ** | Mar'04, May'08 | * | ns | Apr'03 | * |
| Nov'00, Mar'04 | ns | ns | Nov'01, Mar'04 | *** | ns | Jan'03, Jun'07 | ns | ns | Mar'04, Apr'09 | *** | ns | Nov'03 | *** |
| Nov'00, Nov'04 | ns | ns | Nov'01, Nov'04 | *** | ns | Jan'03, May'08 | ns | * | Nov'04, Nov'05 | ** | ns | Mar'04 | *** |
| Nov'00, Nov'05 | * | ns | Nov'01, Nov'05 | ** | * | Jan'03, Apr'09 | * | * | Nov'04, Apr'06 | ns | ns | Nov'04 | ns |
| Nov'00, Apr'06 | ns | * | Nov'01, Apr'06 | *** | * | Apr'03, Nov'03 | *** | ns | Nov'04, Jun'07 | ns | ns | Nov'05 | *** |
| Nov'00, Jun'07 | ns | ns | Nov'01, Jun'07 | *** | ns | Apr'03, Mar'04 | ns | ** | Nov'04, May'08 | ns | ns | Apr'06 | ** |
| Nov'00, May'08 | ns | ns | Nov'01, May'08 | *** | ns | Apr'03, Nov'04 | ns | ns | Nov'04, Apr'09 | * | ns | Jun'07 | ns |
| Nov'00, Apr'09 | ** | ns | Nov'01, Apr'09 | *** | ns | Apr'03, Nov'05 | *** | ns | Nov'05, Apr'06 | * | *** | May'08 | ns |
| Mar'01, Nov'01 | *** | ns | Apr'02, Jan'03 | ns | ns | Apr'03, Apr'06 | ns | *** | Nov'05, Jun'07 | *** | ** | Apr'09 | ns |
| Mar'01, Apr'02 | ns | ns | Apr'02, Apr'03 | ** | ns | Apr'03, Jun'07 | * | * | Nov'05, May'08 | *** | ** | | |
| Mar'01, Jan'03 | ns | ns | Apr'02, Nov'03 | *** | ns | Apr'03, May'08 | * | ** | Nov'05, Apr'09 | *** | ** | | |
| Mar'01, Apr'03 | * | ns | Apr'02, Mar'04 | ** | ns | Apr'03, Apr'09 | *** | ** | Apr'06, Jun'07 | ns | ns | | |
| Mar'01, Nov'03 | *** | ns | Apr'02, Nov'04 | ns | ns | Nov'03, Mar'04 | *** | ns | Apr'06, May'08 | ns | ns | | |
| Mar'01, Mar'04 | * | ns | Apr'02, Nov'05 | *** | * | Nov'03, Nov'04 | *** | ns | Apr'06, Apr'09 | ns | ns | | |
| Mar'01, Nov'04 | ns | ns | Apr'02, Apr'06 | ns | ns | Nov'03, Nov'05 | ** | ns | Jun'07, May'08 | ns | ns | | |
| Mar'01, Nov'05 | *** | ns | Apr'02, Jun'07 | ns | ns | Nov'03, Apr'06 | *** | ns | Jun'07, Apr'09 | ns | ns | | |
| Mar'01, Apr'06 | ns | ns | Apr'02, May'08 | ns | ns | Nov'03, Jun'07 | *** | ns | May'08, Apr'09 | ns | ns | | |
| Mar'01, Jun'07 | ns | ns | Apr'02, Apr'09 | ns | ns | Nov'03, May'08 | *** | ns | | | | | |

**Appendix 4** Continued

| SEF 1 - PERMDISP | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | Amphipods | | | Polychaetes | | Amphipods | | | Im-vs-Co | |
| | Im | Co | Im | Co | | Im | Co | Im | Co | | Polychaetes | Amphipods |
| Oct'02, Oct'03 | * | ns | ** | ns | Oct'04, Jun'06 | *** | ** | ns | ** | Oct'02 | ** | ns |
| Oct'02, Oct'04 | ns | ns | ns | ns | Oct'04, Jun'07 | ns | ns | ns | ns | Oct'03 | ns | ** |
| Oct'02, Oct'05 | ns | ns | ** | ns | Oct'04, Jun'08 | ** | ns | ns | ns | Oct'04 | ns | ns |
| Oct'02, Jun'06 | *** | *** | ns | *** | Oct'04, Jun'09 | ns | ns | ns | * | Oct'05 | ns | *** |
| Oct'02, Jun'07 | ns | ** | ns | ns | Oct'05, Jun'06 | *** | *** | ns | *** | Jun'06 | ns | ns |
| Oct'02, Jun'08 | ** | ns | ns | ns | Oct'05, Jun'07 | ns | * | ** | * | Jun'07 | ns | ns |
| Oct'02, Jun'09 | ns | * | ns | * | Oct'05, Jun'08 | ns | ns | ** | ns | Jun'08 | ns | ns |
| Oct'03, Oct'04 | ns | ns | ns | ns | Oct'05, Jun'09 | ns | ns | * | *** | Jun'09 | ns | ns |
| Oct'03, Oct'05 | ns | ns | ns | ** | Jun'06, Jun'07 | *** | * | ns | ** | | | |
| Oct'03, Jun'06 | *** | *** | ns | ** | Jun'06, Jun'08 | *** | *** | ns | *** | | | |
| Oct'03, Jun'07 | ns | * | ** | ns | Jun'06, Jun'09 | ** | ns | ns | ns | | | |
| Oct'03, Jun'08 | * | ns | ** | ns | Jun'07, Jun'08 | ns | ns | ns | ns | | | |
| Oct'03, Jun'09 | ns | * | ns | ns | Jun'07, Jun'09 | ns | ns | ns | * | | | |
| Oct'04, Oct'05 | ns | ns | * | ns | Jun'08, Jun'09 | ns | ns | ns | * | | | |

**Appendix 4** Continued

| SEF 2 - PERMDISP | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | Amphipods | | | Polychaetes | | Amphipods | | | Im-vs-Co | |
| | Im | Co | Im | Co | | Im | Co | Im | Co | | Polychaetes | Amphipods |
| Jun'01, Jun'02 | * | *** | * | ** | Jun'03, Jun'07 | ns | ns | ns | ** | Jun'01 | * | * |
| Jun'01, Jun'03 | ** | ns | ns | ns | Jun'03, Jun'08 | ns | ** | * | *** | Jun'02 | * | ns |
| Jun'01, Jun'04 | *** | ns | **** | ns | Jun'03, Jun'09 | * | ns | ** | ns | Jun'03 | * | * |
| Jun'01, Jun'05 | *** | ns | ** | ns | Jun'04, Jun'05 | ns | ns | * | ns | Jun'04 | ns | ** |
| Jun'01, Jun'06 | **** | ns | ns | ns | Jun'04, Jun'06 | ns | ns | *** | ns | Jun'05 | * | ns |
| Jun'01, Jun'07 | *** | ns | ns | * | Jun'04, Jun'07 | ns | * | ** | *** | Jun'06 | ** | ns |
| Jun'01, Jun'08 | ** | **** | ** | *** | Jun'04, Jun'08 | ns | **** | * | **** | Jun'07 | ** | ns |
| Jun'01, Jun'09 | ns | ns | *** | ns | Jun'04, Jun'09 | ** | ns | * | ns | Jun'08 | *** | **** |
| Jun'02, Jun'03 | ns | **** | * | ** | Jun'05, Jun'06 | * | ns | * | ns | Jun'09 | ns | ns |
| Jun'02, Jun'04 | * | *** | * | * | Jun'05, Jun'07 | ns | ns | ns | ** | | | |
| Jun'02, Jun'05 | * | **** | ns | * | Jun'05, Jun'08 | ns | ** | ns | *** | | | |
| Jun'02, Jun'06 | *** | * | ns | ns | Jun'05, Jun'09 | *** | ns | ns | ns | | | |
| Jun'02, Jun'07 | ns | **** | ns | **** | Jun'06, Jun'07 | * | * | ns | ** | | | |
| Jun'02, Jun'08 | ns | **** | ns | **** | Jun'06, Jun'08 | ** | *** | ns | *** | | | |
| Jun'02, Jun'09 | ns | *** | ns | ns | Jun'06, Jun'09 | **** | ns | * | ns | | | |
| Jun'03, Jun'04 | ns | ns | **** | ns | Jun'07, Jun'08 | ns | ns | ns | ns | | | |
| Jun'03, Jun'05 | ns | ns | ** | ns | Jun'07, Jun'09 | ** | ns | ns | ** | | | |
| Jun'03, Jun'06 | ** | ns | ns | ns | Jun'08, Jun'09 | * | ns | ns | *** | | | |

**Appendix 5** Results of the *a posteriori* univariate PERMANOVA pair-wise comparisons for the significant 'Im-vs-Co x Ti' interaction term for the polychaete and amphipod taxa that contributed most (in terms of number of individuals) to the high dissimilarity of samples between impacted and control plots recorded from the northeastern farm (NEF), southeastern 'Farm 1' (SEF 1), and southeastern 'Farm 2' (SEF 2) during the study period (Chapter 7). Level of significant set at 0.05. Ampeliscidae (Amp), Capitellidae (Cap), Caprellidae (Capr), Dorvilleidae (Dor), Glyceridae (Gly), Hesionidae (Hes), Lysianassidae (Lys), Maeridae (Mae), Maldanidae (Mal), Paraonidae (Par), Photidae (Pho), Phoxocephalidae (Phox), Syllidae (Syl), Urothoidae (Uro), ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

| NEF | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | | | | Amphipods | | |
| | Cap | Dor | Hes | Mal | Par | Lys | Phox | Uro |
| Nov'00 | ns | - | nt | ns | - | ns | ns | - |
| Mar'01 | ns | - | ns | ns | - | ns | ns | - |
| Nov'01 | > ** | - | < ** | ns | - | ns | < ** | - |
| Apr'02 | < ** | - | < *** | < ** | - | ns | < *** | - |
| Jan'03 | > * | - | < *** | < ** | - | ns | < *** | - |
| Apr'03 | ns | - | ns | < * | - | < * | < * | - |
| Nov'03 | > ** | - | < ** | < ** | - | ns | < *** | - |
| Mar'04 | ns | - | < *** | < *** | - | < ** | < *** | - |
| Nov'04 | ns | - | < ** | ns | - | < * | ns | - |
| Nov'05 | ns | - | < ** | < *** | - | < *** | < *** | - |
| Apr'06 | ns | - | < ** | ns | - | ns | < *** | - |
| Jun'07 | ns | - | < *** | ns | - | ns | < *** | - |
| May'08 | < ** | - | < * | < *** | - | < *** | < *** | - |
| Apr'09 | < * | - | < ** | ns | - | ns | < ** | - |

| SEF 1 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | | | Amphipods | | | | |
| | Cap | Gly | Mal | Par | Amp | Capr | Lys | Pho | Uro |
| Oct'02 | - | ns | < * | - | ns | - | - | ns | > * |
| Oct'03 | - | ns | ns | - | < * | - | - | < * | ns |
| Oct'04 | - | ns | ns | - | ns | - | - | ns | ns |
| Oct'05 | - | ns | ns | - | < *** | - | - | < *** | ns |
| Jun'06 | - | < ** | < * | - | < ** | - | - | < *** | ns |
| Jun'07 | - | < ** | < ** | - | < * | - | - | < *** | ns |
| Jun'08 | - | ns | ns | - | ns | - | - | ns | ns |
| Jun'09 | - | ns | ns | - | ns | - | - | ns | ns |

| SEF 2 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Polychaetes | | | Amphipods | | | | | | |
| | Gly | Par | Syl | Amp | Capr | Lys | Mae | Pho | Phox | Uro |
| Jun'01 | - | - | - | - | - | ns | - | ns | ns | - |
| Jun'02 | - | - | - | - | - | ns | - | ns | ns | - |
| Jun'03 | - | - | - | - | - | ns | - | ns | ns | - |
| Jun'04 | - | - | - | - | - | ns | - | ns | ns | - |
| Jun'05 | - | - | - | - | - | ns | - | ns | ns | - |
| Jun'06 | - | - | - | - | - | ns | - | < *** | < ** | - |
| Jun'07 | - | - | - | - | - | < *** | - | < *** | < * | - |
| Jun'08 | - | - | - | - | - | ns | - | < * | ns | - |
| Jun'09 | - | - | - | - | - | ns | - | ns | ns | - |