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Assessment of the impact of Atlantic bluefin tuna farming on Adriatic benthic habitats by analysing macroinvertebrate assemblage structure at family level

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Abstract

The rapidly expanding Atlantic bluefin tuna fattening industry is characterized by high stock densities and a high input food biomass in the form of whole bait seafood. The environmental impact of this activity must be effectively monitored within a proper sustainable development framework, to address concerns about the potential adverse effects. However, evaluation of monitoring tools for tuna farming has received less attention than other activities. Based on the principles of key taxa (Pocklington & Wells, 1992), we tested the potential use of changes in benthic macroinvertebrate assemblages, polychaetes and amphipods for this purpose. Applying a non-parametric multivariate approach for monitoring the impact of this aquaculture activity on the benthic habitat, we checked for correlations with the physicochemical environmental variables of the sediment. A hierarchical spatial design was followed, using multiple controls. Amphipods and polychaetes showed dissimilarities between impacted and control locations, with significant differences for total assemblage structure at a taxonomic level of families. Total nitrogen (TN) and total sulfur (TS) concentrations were the variables best associated with these changes for amphipods, and d ^{13}C and total phosphorus (TP) were the best for polychaetes. However, total free sulfides (TFS) and TP were the chemical variables that best indicated the effects on sediment. Using this approach, surrogating the whole benthic assemblage to a single taxocene, our data suggest that monitoring tuna farming impact by comparing the changes in amphipod and polychaete assemblages at family level could be an optimal procedure with an excellent cost/benefit ratio.

Keywords: Mediterranean Sea; Tuna farming; Aquaculture impact; monitoring program.

Introduction

Mandatory or voluntary environmental monitoring of aquaculture activities in coastal areas is one of the most effective tools within a proper sustainable development framework (FAO, 2009). Despite this, monitoring programs are highly inconsistent between countries, which results in many different approaches and interpretations of their data. However, it is generally accepted that monitoring of benthic habitats is necessary to assess the negative environmental impacts; consequently macrozoobenthic communities are widely selected as a useful indicator because they live in close association with bottom substrata (Karakassis *et al*., 2000; Tomassetti & Porrelo, 2005; Fernandez-Gonzalez *et al*., 2013). The sea-bed below cages is where contaminant build-up and low-oxygen conditions due to fish farming activities are frequently most critical. During the last decade, after the publication of the European Water Framework Directive (WFD, 2000/60/EC), assessment of direct changes in benthic macroinvertebrate assemblages has been replaced by the development of benthic biotic indexes, with the aim of standardizing the methodologies used to typify and monitor the environmental quality of European water bodies (Van Hoey *et al*., 2010; Birk *et al*., 2012). The application of such autecology-based metrics can be questioned for many reasons outlined in Dauvin *et al*. (2012), for example the difficulty in assigning tolerance/sensitivity levels to each taxon (Carvalho *et al*., 2006; Labrune *et al*., 2012), and/or misclassification of their ecological quality status (Quintino *et al*., 2006; Callier *et al*., 2008). In contrast, using the taxonomy-based metrics approach, environmental changes are directly linked to changes in the structure of macroinvertebrate assemblages, depending on their ecological requirements (Clarke, 1993; Cabana *et al*., 2013). These, in turn, are based only on their taxonomic identification and often on the macrobenthic abundance. Additionally, many monitoring programs have focused on the key taxocene as surrogates of the overall changes in the entire macrobenthic community (Arvanitidis *et al*., 2009), which reduces the monitoring cost and time.

Although species are considered the taxonomic level that provides the most reliable information, their identification is a time-consuming process requiring considerable taxonomic expertise, and is subject to errors. This process is necessary for the calculation of many of the biotic indices such as AMBI (AZTI Marine Biotic Index; Borja & Muxika, 2005), BENTIX (Benthic Index; Simboura & Zenetos, 2002), or MEDOCC (Mediterranean Occidental Index; Pinedo *et al*., 2015), and for the identification of the entire benthic community structure or its taxocenose. This results in an obstacle for calculation of benthic biotic indices (Somerfield & Clarke, 1995). Identifying organisms at higher taxonomic levels may help to overcome these problems as it can provide sufficient information for an optimal environmental assessment and the subsequent management of fish farming (Forrest & Creese, 2006; Naser, 2010), by minimizing the time required and financial cost. This approach was defined by Ellis (1985) as taxonomic sufficiency and widely applied for macroinvertebrate studies. It is already recommended for seabream and seabass aquaculture monitoring programs (Aguado *et al.,* 2015).

The tuna fattening industry has expanded rapidly in the Mediterranean Sea (Miyake *et al*., 2003), and also in Australia, Mexico and Japan; world production of farmed tuna reached 36350 t in 2014. The main capture-based aquaculture producers of Atlantic bluefin tuna in the Mediterranean Sea are in Spain, Malta and Croatia, which together accounted for more than 14,500 t in 2014 (Tveteras *et al.,* 2015). As in all intensive rearing systems, tuna farming requires high stocking densities and a high input of food biomass in the form of fish and cephalopod bait caught fresh in the locality or imported frozen from abroad. Hence, there have been growing concerns about the potential adverse environmental effects of the many tuna farms in the Mediterranean, particularly because of the deleterious effects of uneaten feed and fish feces on benthic communities (Metian *et al*., 2014). However, evaluation of monitoring tools for the more recent tuna farming has received less attention than other activities, such as mollusk production, or seabream and seabass farming, but several authors have assessed its environmental impact following various approaches (Aksu *et al*., 2010; Vezzulli *et al*., 2008; Mangion *et al*., 2014).

Tuna farming in Croatia is becoming a growing industry in coastal environments. In 2015, it produced 2603 t of bluefin tuna (http://www.mps.hr/ribarstvo/default.aspx?id=14). Based on key taxon principles (Pocklington & Wells, 1992) within a taxonomic sufficiency approach to fish farming monitoring programs, we assessed the impacts of Croatian tuna farming on macroinvertebrate assemblages following a non-parametric multivariate analysis approach. The experimental design included a hierarchical spatial design and multiple controls for correct spatial replication (Underwood, 1994). We obtained information at the family level for selected taxa of polychaete and amphipod assemblages (Mangion *et al.*, 2014), and applied multivariate analyses of ecological communities based on measures of dissimilarity using the Bray-Curtis index (Clarke, 1993; Anderson, 2001; Clarke & Gorley, 2006). The present study aimed to (i) evaluate the input of organic matter and nutrients from tuna farming in Croatia, compared with control locations, and ii) assess the sensitivity of the relevant benthic macroinvertebrate assemblages (amphipods and polychaetes) at family level, correlating that to a number of sediment physicochemical variables.

Material and Methods

Study site and sampling design

A tuna farming facility in Vela Grška Bay (43°17′15.07″N, 16°28′56.48″E), south-west of the island of Brač, Adriatic Sea, comprising six 50-meter cages at 35 m depth, was sampled in this study. Two bays unaffected by aquaculture activities and far away from other anthropogenic activity as possible were selected as control locations, within a distance of 2 and 3.2 km from the fish farm (Fig. 1). Three sampling locations were located between 100 and 350 m from the coastline. At each of three locations (one farm and two control locations), three random sites were allocated, sampling three replicates at each site to collect physicochemical and macroinvertebrate data, in summer and winter conditions (September 2013 and March 2014).

Benthic sampling

The granulometric composition of the sediment samples was determined from benthic samples using the Bouyucos method (Buchanan, 1984). Sediment type was classified according to Folk (1954). Total free sulfide (TFS) content was measured in a sulfide antioxidant buffer solution and ascorbic acid, using a silver/sulfide half-cell electrode following the method described by Wildish *et al*. (1999). Total carbon (TC), total hydrogen (TH), total sulfur (TS), total nitrogen (TN) and total phosphorus (TP) were determined with an elemental auto-analyzer (LECO 932). While stable isotopes ^{13}C and ^{15}N were measured in sediments from traps and sea bottom, only the traps showed sufficient nitrogen content (at least 0.1%) to quantify the ^{15}N-isotope. The isotope composition was measured using an EA-IRMS analyzer (Thermo Finnigan) in continuous flow configuration connected to a stable ratio mass spectrometer Delta Plus, 0.1‰ being the analytical precision. The ratios of ^{15}N/^{14}N and ^{13}C/^{12}C were expressed as the relative per mill difference (delta: d, ‰) between the sample and conventional standards (air for N; Pee Dee belemnite limestone carbonate for C). Macrofauna samples were collected by scuba diving, using 20x20 cm quadrats, at depths between 30 and

Fig. 1: Location of the studied area. (Map: Google Earth. Image © 2017 TerraMetrics/CNES /Airbus).

40 m. Quadrats were randomly deployed, covered with a 0.5 mm mesh bag, collecting the first 5 cm of the substrate with a scoop. Samples were sieved through a 0.5 mm mesh and the retained residues preserved in a 10% formalin seawater solution. In the laboratory, the macrofaunal individuals were sorted, identified to family level and counted.

Sedimentation rate estimations

To estimate sedimentation rates and the organic effluent from tuna farming, standard sediment traps (12 cm diameter x 100 cm long) were deployed at 30 meters depth: two at the farm, and two more at one km from the farm for comparison with natural sedimentation rates. Samples were taken during two different periods in summer and two in winter, on approximately 30 days in summer, and 60 days in winter, to collect a sufficient quantity of organic matter for stable isotope and CHNS measurements. Due to weather conditions resulting in the loss of some samples, only two samples were obtained at control and farm sites in summer and three at the farm in winter. All the samples (n=4) were obtained during winter at the control location.

Experimental design and statistical analysis

Triangular similarity matrices were calculated using the Bray-Curtis similarity coefficient (Clarke & Gorley, 2006), after transforming the data by square root to weight the contributions of common and rare species (Clarke, 1993). Non-metric multidimensional scaling (nMDS) was used as the ordination method to depict patterns of the macrobenthic community and its taxocene, showing the stress value (Kruskal & Wish, 1978). The SIMPER routine determined the contribution of each taxon and family to the dissimilarity between treatments (farm and controls). After SIMPER analysis, polychaetes and amphipods were selected from the overall assemblage to investigate the effects on the assemblage structure as a whole (Clarke & Gorley, 2006). The data were submitted to permutational multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis dissimilarities (Anderson, 2001). This generated 4999 random permutations of residuals under the full model, with appropriate units as required by the design (Anderson *et al*., 2008). To explore the environmental variables that best explain the changes in macroinvertebrate assemblages we applied the routine Distance-based linear model (DistLM). The relationship between a multivariate data cloud and predictor variables was tested, based on a resemblance matrix. This used permutations rather than the restrictive Euclidean distance and normality assumptions that underlie the standard approach to linear modeling following Anderson *et al*. (2008). The Akaike information criterion (AIC) was used to choose the best model from all possible combinations of variables. All the multivariate analyses were run using PRIMER 6 v.6.1 (PRIMER software; Clarke & Gorley, 2006) and PERMANOVA+ v.1.0 add-on package (Anderson *et al*., 2008), developed by Plymouth Marine Laboratory (UK). The experimental design considered four factors: Season (fixed with two levels; summer and winter); Control/Impact (fixed with two levels); Location, random and nested in Control/Impact but asymmetrical because they consisted of one farm location and two control locations; and Site, random and nested in all mentioned factors. Multivariate procedures were applied for the analysis of macrofauna assemblage matrixes, considering each taxon as a variable and its abundance as an attribute.

Results

The sedimentation rates were higher at the farm compared to controls, particularly during the winter season. The quantity of organic matter collected in traps placed at control sites was 4.20 ± 0.27 and 9.78 ± 1.78 g/m^2/day (Fig. 2), in summer and winter respectively. At farm sites, the sedimentation rate was 22.73 ± 1.03 and 32.20 ± 1.9 g/m^2/day in summer and winter respectively, showing marginally significant differences (Im: $p = 0.089$; Table 1). The stable isotope signatures from sediment traps at fish farm and control locations were well differentiated by N (Fig. 3), showing the controls had more depleted d^{15}N signatures. Furthermore, d^{13}C separated winter and summer samples, especially those from the farm, which were the most depleted. This organic sediment had a significantly higher TC content in summer, being slightly higher at control sites, while values at farm sites in winter were remarkably higher compared to control sites (Table 1, SexIm $p = 0.021$, Fig. 4). As for TH, a significantly higher percentage was detected at the farm in summer (Table 1, SexIm $p = 0.003$, Fig. 4), as well as a higher TN percentage during summer (Table 1, SexIm $p = 0.003$, Fig. 4). In both cases, during winter seasons concentrations were higher at the farm, but without significant differences. No statistical differences were found for TS.

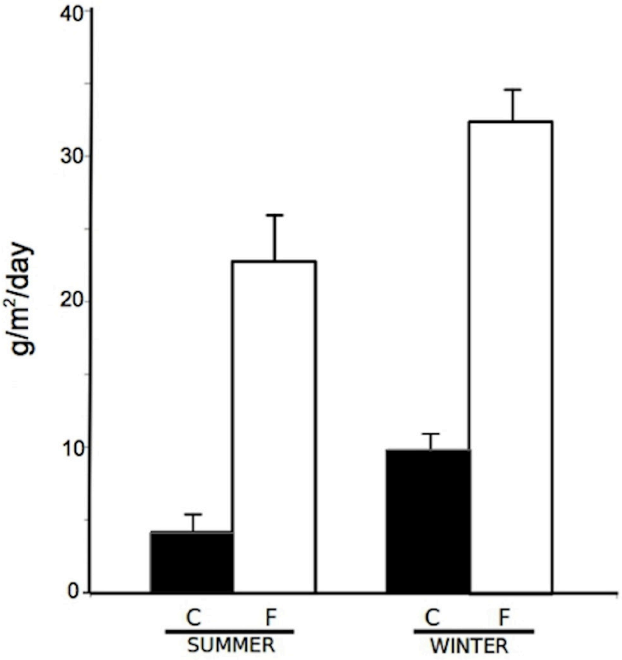

Fig. 2: Sedimentation rates of total organic matter (mean ± SE) estimated using sediment traps at control (C) and farm (F) locations, during summer and winter seasons.

Granulometric analysis showed that all sediments in the study were sandy, with at least 60% sand and gravel in their composition. There were no statistical differences between seasons with respect to the proportion of TC in the sea-bottom sediments collected at farm and control sites (Table 1; Fig. 5), showing very low concentrations in winter. Values ranged between 11.47 and 11.61% in summer and 11.02 and 11.27 % during winter. In summer, TH and TN contents were found to be significantly

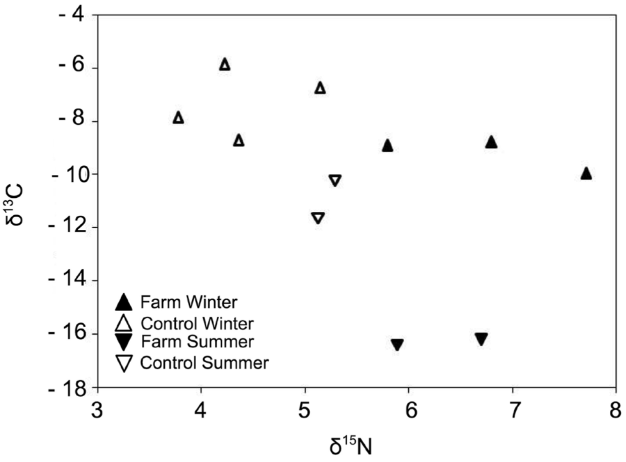

Fig. 3: Plot of delta ^{13}C *versus* delta ^{15}N (mean ± SE) for organic matter from sediment traps deployed below tuna farms and control locations, during summer and winter seasons.

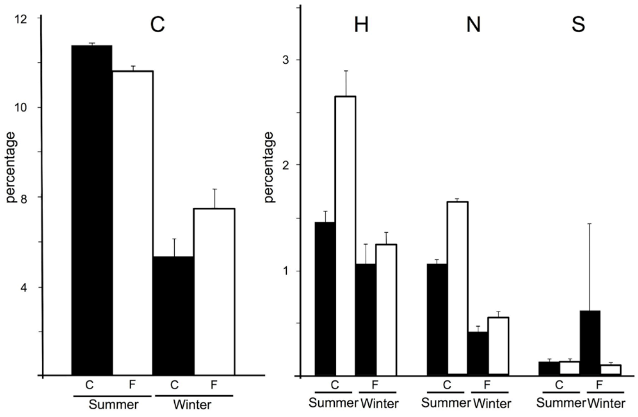

Fig. 4: Results for the percentage (mean ± SE) of total carbon (C), total hydrogen (H), total nitrogen (N) and total sulfur (S) from the organic matter collected by means of sediment traps at control (C) and farm (F) locations, in summer and winter.

Table 1. PERMANOVA results of chemical variables from traps and sediment samples. Df = degrees of freedom, MS= mean square, P = level of significance.

Traps		**OM**		**TC**		**TH**		**TN**		**TS**	
	df	MS	P	MS	P	MS	P	MS	P	MS	P
Se	1	0.131	0.719	8.844	0.002	5.36	0.001	7.87	0.001	0.465	0.583
Im	1	3.40	0.089	0.039	0.332	3.15	0.001	1.541	0.001	0.640	0.609
SexIm	1	0.092	0.759	0.380	0.021	1.68	0.003	0.359	0.003	0.677	0.601
Residual	7	0.919		0.031		0.076		0.026		1.08	

Sediment		**TC**		**TH**		**TN**		**TS**		**TP**		**TFS**		**$d^{13}C$**	
	df	MS	P	MS	P	MS	P	MS	P	MS	P	MS	P	MS	P
Se	1	17.82	0.178	1.840	0.465	0.588	0.503	0.582	0.807	0.039	0.153	9.78	0.234	3.94	0.173
Im	1	0.079	1	14.73	0.001	32.17	0.001	0.749	0.879	35.58	0.001	12.9	0.001	6.25	0.641
Lo(Im)	1	1.139	0.18	1.294	0.301	0.778	0.337	0.030	0.989	0.027	0.859	0.693	0.476	11.89	0.001
SexIm	1	1.107	0.454	0.049	0.916	0.041	0.832	5.337	0.497	0.039	0.163	0.028	0.935	3.79	0.154
SexLo(Im)	1	1.061	0.189	1.920	0.227	0.701	0.366	4.69	0.099	0.004	0.958	3.22	0.115	0.062	0.84
Si(SexLo(Im))	12	0.577	0.666	1.153	0.031	0.789	0.007	1.473	0.005	0.832	0.001	1.18	0.002	1.03	0.005
Residual	36	0.717		0.536		0.257		0.494		0.203		0.292		0.462	

higher at farm sites in spite of the wide spatial variability, increasing 45 % and 35 %, respectively (Table 1; Fig. 6). This pointed to the influence of farm activity. TS also exhibited a very high variability without significant differences (Table 1; Fig. 6). The TP content in farm sediment showed a statistical difference from controls, with higher values (77 and 81.8 mmol/kg in summer and winter respectively) at the farm (Table 1; Im $p = 0.001$) than the average around 8.09 mmol/kg at control locations (Fig. 7).

During both seasons, the total value of total free sulfides (TFS) was significantly higher at farm sites (Table 1; $p < 0.01$), where the average values were 4.15 ± 0.51 and 2.74 ± 0.2 mg/l in summer and winter, respectively. At control sites, the average concentration was 2.33 ± 0.35 and 0.95 ± 0.17 mg/l, in summer and winter respectively (Fig. 8). Even though sandy (therefore well-oxygenated) sediments were present at all sites, higher concentrations of TFS were significantly more frequent at farmed sites. Also in this farm environment, the average $d^{15}N$ ratio was 8.87 ± 0.9 and 5.61 ± 0.23, in summer and winter respectively. Owing to the very low N concentration, it was only possible to measure five farm samples in summer and nine in winter, and insufficient N isotopes were obtained in control samples. No differences were observed in $d^{13}C$ between farm and control sites, showing a wide spatial variability on the scale of locality and

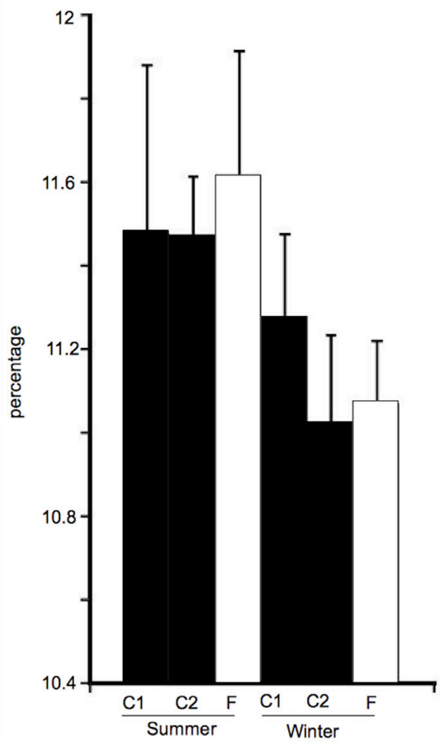

Fig. 5: Mean percentage (mean ± SE) of carbon content of sediments in summer and winter at control (C1 and C2) and farm (F) locations.

sites [Table 1; Lo(Im) $p = 0.01$; Si(SexLo(Im)] $p = 0.05$; Fig. 9).

Seventeen macroinvertebrate taxa (taxonomic level of phyla and class) were identified in the samples, the most abundant being polychaetes (61.5%), followed by amphipods (26.9%) and bivalves (4.6%). Polychaetes were represented by 28 families, 16 at farm and 26 at control locations. Amphipods showed a lower number of families, with a total number of 16, with only 5 and 14 in impacted and control samples, respectively. SIMPER analysis indicated an average dissimilarity of 46.09% between control and impact locations, amphipods (17.7%) and polychaetes (14.97%) being the most important taxa inducing dissimilarity (Table 2). Significant dissimilarity was observed between control and farm locations, supported by the PERMANOVA significance test ($p = 0.02$, Table 3), with a high spatial variability across sites ($p = 0.003$), whereas no significant differences were found between seasons.

Amphipods contributed the most to dissimilarities (SIMPER, total dissimilarity 99.14%, Table 4); with the main families being Nuuanuidae, Oedicerotidae and Ischyroceridae (SIMPER; 42.1, 14.5 and 10.8% of total dissimilarity, respectively). Of these, the greatest was basically due to the high presence of Nuuanuidae at the farm

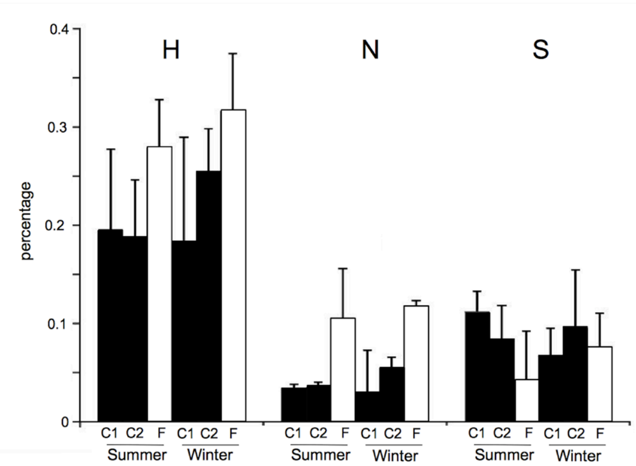

Fig. 6: Results of the percentage (mean ± SE) total hydrogen (H), total nitrogen (N) and total sulfur (S) content in sediments during summer and winter at control (C1 and C2) and farm (F) locations.

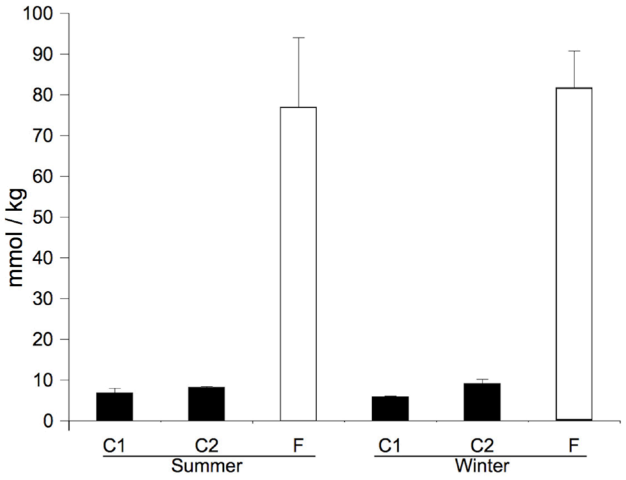

Fig. 7: Total phosphorus (TP) content (mean ± SE) in the sediments of the two control locations C1 and C2, and the tuna fish farm (F).

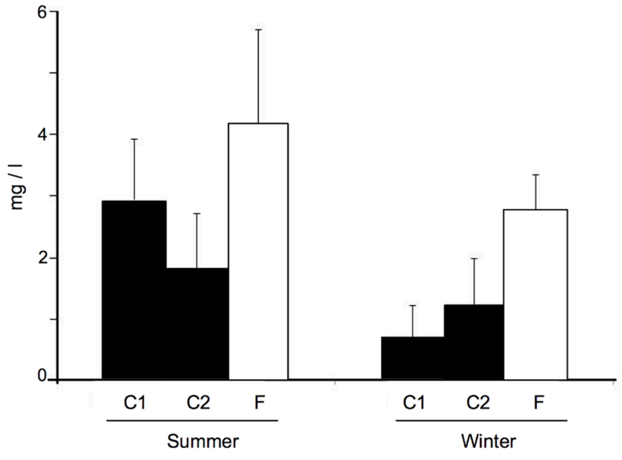

Fig. 8: Total free sulfides (TFS) in mg/l (mean ± SE) in sediment, at each control (C1 and C2) and fish farm location (F), during summer and winter season.

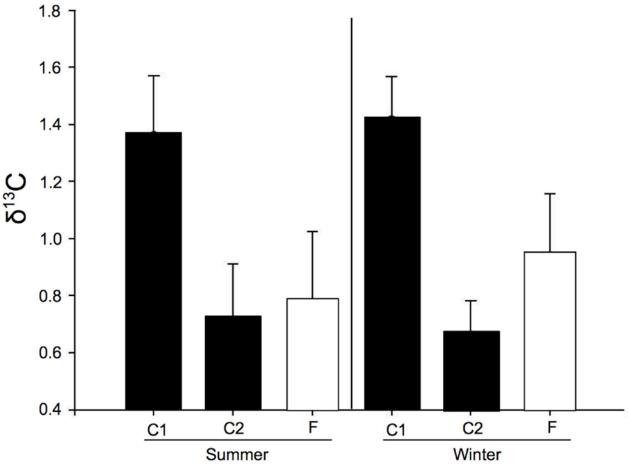

Fig. 9: Delta ^{13}C (mean ± SE) in the sediment samples at control locations (C1 and C2) and the farm location (F) in summer and winter seasons.

location. Non-metric multidimensional scaling (nMDS) (Fig. 10a) revealed marked dissimilarities between control and farm sites that were supported by PERMANOVA (Table 5; p = 0.025), showing a significant effect of season on the impact of fish farming (Se x Im, p = 0.016), despite the high spatial variability at site level (p = 0.0034).

Polychaetes also showed a wide dissimilarity between farm and control sites (SIMPER, total = 82.57%; Table 6). The main families responsible for differences between farm and control locations, largely present in the farm sediments, were Spionidae, Nereididae and Capitellidae (SIMPER; 23.6, 17.3 and 12.6% of total dissimilarity respectively). Contrastingly, Dorveilleidae, Syllidae, Oweniidae and Pisionidae were most abundant at control locations, but with less weight (between 3 and 5% of total dissimilarity). The nMDS showed a very clear discrimination between control and farm samples (Fig. 10b), statistically confirmed by PERMANOVA (Table 7; Im, p = 0.005), in spite of the high spatial heterogeneity between seasons (Se x Lo(Im), p = 0.0034) and across sites (Si(SexLo(Im)), p = 0.0144).

Of all sediment predictor variables (TP, TC, TH, TN, TS, TFS and $d^{13}C$), DistLM analysis indicated that TN, TS and $d^{13}C$ were the variables that best explained changes in amphipod assemblages (AIC = 429.64). For polychaetes, inorganic phosphorus (IP) was the most important (AIC = 410.8).

Table 2. Summary of SIMPER dissimilarity results for macroinvertebrate assemblages between control (C) and farm (F) locations. AD = Average dissimilarity, Av.Abund=average macroinvertebrate abundance, Contrib.%= percentage contribution to dissimilarity for each taxon, Cum.%= cumulative percentage of dissimilarity.

Families (AD= 46.09)	F Av.Abund	C Av.Abund	Contrib.%	Cum.%
Amphipoda	1.59	1.03	17.70	17.70
Polychaeta	2.55	1.71	14.97	32.67
Bivalvia	0.83	0.89	11.12	43.79
Tanaidacea	0.29	0.23	7.79	51.58
Branchiostomidae	0.06	0.39	7.27	58.85
Gasteropoda	0.26	0.15	5.30	64.15
Leptostraca	0.35	0.06	5.26	69.41
Echinoidea	0.17	0.23	5.22	74.63
Copepoda	0.22	0.14	4.95	79.57
Sipunculida	0.07	0.23	4.27	83.84
Cumacea	0.06	0.21	3.95	87.79
Ophiuroidea	0.07	0.13	2.81	90.60

Table 3. PERMANOVA results of macroinvertebrate assemblage. Df= degrees of freedom, MS= mean square, P= level of significance.

Source	df	MS	Pseudo-F	P(perm)
Se	1	3105.9	1.5952	0.262
Im	1	11882	7.7098	0.024
Lo(Im)	1	1502.7	1.2397	0.336
SexIm	1	2440.7	1.2559	0.376
SexLo(Im)	1	1908.2	1.5742	0.165
Si(SexLo(Im))	12	1220.7	1.5591	0.003
Residual	35	27404	782.97	

Discussion

Amphipods and polychaetes could be considered as indicator taxa, identified at family level, for monitoring the environmental effects of tuna farming. Analyzing the changes in macroinvertebrate assemblages using a non-metric multivariate analysis seems to be a robust

Table 4. Summary of SIMPER dissimilarity results for amphipod assemblages between control (C) and farm (F) locations. AD= Average dissimilarity; Av.Abund.= average macroinvertebrate abundance, Contrib.%= percentage contribution to dissimilarity for each taxon, Cum.%= cumulative percentage of dissimilarity.

Families (AD= 99.14)	F Av.Abund.	C Av.Abund.	Contrib.%	Cum.%
Nuuanuidae	3.14	0.00	42.12	42.12
Oedicerotidae	0.00	0.47	14.54	56.67
Ischyroceridae	0.58	0.16	10.88	67.55
Dexaminidae	0.00	0.21	5.61	73.16
Stenothoidae	0.11	0.03	3.62	76.78
Photidae	0.21	0.03	3.11	79.89
Caprellidae	0.00	0.11	3.02	82.92
Aoridae	0.06	0.03	2.81	85.72
Maeridae	0.06	0.00	2.66	88.38
Corophiidae	0.00	0.06	2.48	90.86

Table 5. PERMANOVA results of amphipod assemblages. Df= degrees of freedom, MS= mean square, P= level of significance.

Source	df	MS	Pseudo-F	P(perm)
Se	1	3930.3	1.9654	0.1514
Im	1	24848	4.2135	0.0258
Lo(Im)	1	5757	1.3725	0.1952
SexIm	1	8985.9	4.4467	0.0168
SexLo(Im)	1	1983.2	0.47279	0.906
Si(SexLo(Im))	12	4224.3	1.5627	0.0034
Residual	35	2703.2		

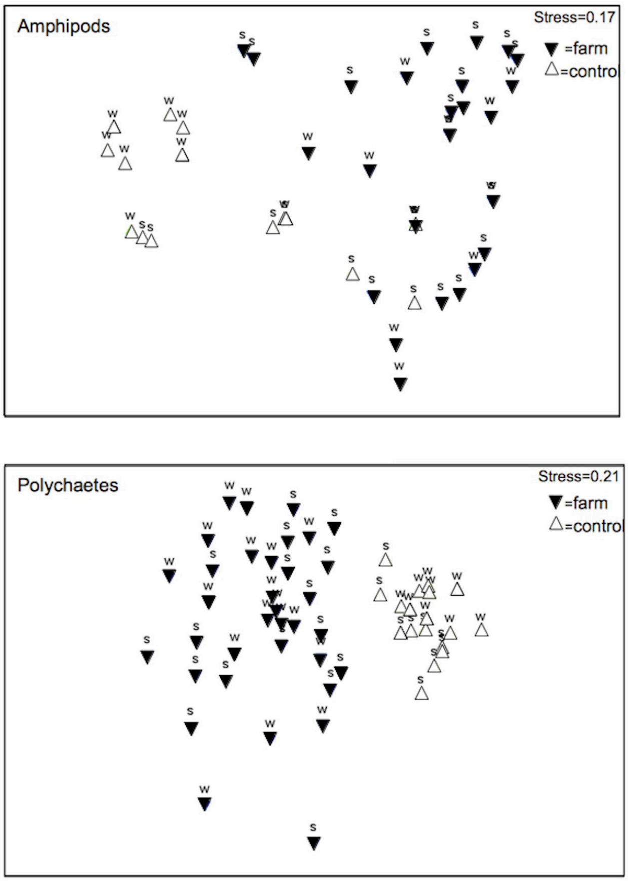

Fig. 10: Non-metric multidimensional scaling ordination (MDS) analyses of the Bray-Curtis similarity of square-root transformed abundance data for each amphipod and polychaete family, during summer (S) and winter (W) at control and farm locations.

Table 6. Summary of SIMPER dissimilarity results for polychaete assemblages between control (C) and farm (F) locations. AD= Average dissimilarity, Av.Abund.= average macroinvertebrate abundance, Contrib.%= percentage of contribution on dissimilarity for each taxon, Cum.%= cumulative percentage of dissimilarity.

Families (AD=82.57)	F Av.Abund.	C Av.Abund.	Contrib.%	Cum.%
Spionidae	5.18	0.96	23.64	23.64
Nereididae	3.73	0.03	17.30	40.94
Capitellidae	2.87	0.38	12.67	53.61
Dorvilleidae	0.66	0.82	5.20	58.81
Syllidae	0.51	0.99	4.76	63.57
Oweniidae	0.00	0.75	3.87	67.43
Pisionidae	0.00	0.73	3.57	71.00
Phyllodocidae	0.64	0.15	3.49	74.49
Paraonidae	0.00	0.51	2.80	77.30
Sabellidae	0.00	0.46	2.77	80.07
Glyceridae	0.25	0.44	2.36	82.43
Lumbrineridae	0.00	0.44	2.25	84.68
Cirratulidae	0.48	0.06	2.16	86.84
Magelonidae	0.06	0.32	1.66	88.50
Eunicida	0.00	0.28	1.28	90.05

Table 7. PERMANOVA results for polychaete assemblages. Df= degrees of freedom, MS= mean square, P= level of significance.

Source	df	MS	P(perm)
Se	1	4660.1	0.729
Im	1	39315	0.005
Lo(Im)	1	2744.6	0.3314
SexIm	1	2944.6	0.8848
SexLo(Im)	1	7047.3	0.0034
Si(SexLo(Im))	12	2379.6	0.0144
Res	35	1726.8	

tool for defining environmental impact due to fish farming. Such statistical methods avoid issues derived from non-normal data, or arbitrary, discontinuous or questionable scales, and the assumption of linear relationships among variables (Clarke, 1993; Anderson, 2001; Anderson *et al.*, 2008). Taxonomic resolution at family level provides enough information to discriminate environmental status in the managment decision process, reducing monitoring costs. Faunal assemblages were associated with the rate of matter sedimentation from tuna farming activities, while TP, TN, TS and d ^{13}C were the chemical variables associated with their structure. Additionally, TP and TFS were the chemical variables that best reflected the effects of tuna farming on soft sediments.

Several studies on the potential environmental impacts of tuna farming have been carried out in the Mediterranean (Aksu *et al.*, 2010; Matijević *et al.*, 2006; Mangion *et al.*, 2014; Moraitis *et al.*, 2013; Vezzulli *et al.*, 2008; Vita *et al.*, 2004), suggesting that the most important factor is sedimentation of uneaten bait, which could lead to anoxic conditions. However, when there is a proper feeding management strategy at the facility, the input of organic matter into the benthos from uneaten bait can be relatively low. Furthermore, tuna feces are more soluble than those of other farmed fish species, dispersing more readily while settling through the water column, hence resulting in less deposition of particulate waste beneath tuna cages (Vita *et al.*, 2004). In contrast, fish bones from uneaten baitfish accumulate and decompose on the seabed over a longer period (Mangion *et al.*, 2014), having a press perturbation on the sediment, in increasing P and N concentration rather than organic matter. Aguado *et al.* (2004) also reported low P assimilation efficiency (ranging from 0.46–0.66) in Atlantic bluefin tuna fed with wild-caught fish prey (mackerel, herring, pilchard and gilt sardine), which would increase the input of P around fish farms. The seabed in the vicinity and beneath tuna farms is therefore subject to high-sediment organic loading resulting from the high biomass of farmed fish, their overfeeding regime, and the build-up of uneaten baitfish and feces. Therefore P concentration notably increases. In general, a decomposing mass of organic matter on the seabed below tuna would cages fosters microbial activity, leading to low redox sediment conditions (Holmer *et al.*, 2008). In the present study, sedimentation rates and increased TP and TFS values showed similar patterns. However, they appear not to overload the carrying capacity of soft sediment, because these TFS values varying between 2 and 5 mg/l are characteristic of sediments in very good condition (Hargrave *et al.*, 2008). Nevertheless, TP content was higher when compared with the controls and the usual values previously recorded in the Adriatic Sea off the Croatian coast (17.2-27.6 mmol/kg; Matijević *et al.*, 2008). The high values in winter may be related to farming management practices. In the Mediterranean, farming companies normally start stocking tuna in cages in late spring, while in Croatia and Malta in particular they often extend the caging season into late summer (Ottolenghi, 2008). The greater feed input would increase growth during winter, explaining the higher sedimentation rates in this period. However, sea currents play an important role in dispersing particulate matter to benthic habitats, with a potential increase in dispersion rate in winter due to stronger hydrodynamics (Aksu *et al.*, 2010).

These mild environmental changes due to tuna farming still have the potential to influence the structure of macroinvertebrate assemblages. The use of polychaetes as environmental indicators has been well described (e.g. Giangrande *et al.*, 2005). They are found in all benthic habitats and are highly sensitive to different types of soft-sediment disturbance such as organic loading, as demonstrated through monitoring the impact of farming on the benthic habitat (e.g. Tomassetti & Porrello, 2005; Lee *et al.*, 2006; Martinez-Garcia *et al.*, 2013). Normally organic matter induces significant changes in polychaete abundance, species richness and diversity in the vicinity of fish farms. In contrast, in the present study TP was the most important factor, showing a clear impact gradient. This is probably linked to the above-mentioned accumulation of fish bone remains. Amphipods are an important group of benthic fauna in terms of abundance and diversity and are commonly used as indicator because of their greater sensitivity to pollution compared to other crustacea (Sanchez-Jerez & Ramos-Espla, 1996; Gomez-Gesteira & Dauvin, 2000; De-la-Ossa-Carretero *et al.*, 2011; Carvalho *et al.*, 2012). Consequently, previous studies (Fernandez-Gonzalez & Sanchez-Jerez, 2011; Fernandez-Gonzalez *et al.*, 2013) recommend the use of this taxon as a biological indicator of changes potentially resulting from sea bream and sea bass farming activities. Similarly, our results corroborate the usefulness of amphipods as an indicator for monitoring how tuna farming activities affect benthic habitats. Indeed, an important reduction in the amphipod families at the impact location could be interpreted as an impairment of ecosystem health.

The three families of polychaetes that showed more weight for differences between impact and control locations are widely accepted as characteristic of polluted zones, in particular Spionidae (Dean, 2008). Nereididae includes pollution indicators (Méndez *et al.*, 1998) and species that live in impacted areas (Pagliosa, 2005). Capitellidae is also well known for its tolerance to pollution (Giangrande *et al.*, 2005). Several authors have analyzed changes in different polychaete families in response to farming activities, obtaining similar results. Martinez-Garcia *et al.* (2013) showed an increase in tolerant families such as Capitellidae and Glyceridae, accompanied by a reduction in families sensitive to fish-farm wastes and thus indicative of non-polluted conditions, including Maldanidae and Paraonidae. Mangion *et al.* (2014) found changes in Capitellidae and Paraonidae, so the relative importance of these families could change depending on biogeography, seasonal or local environmental variations such as in granulometry. Moreover, a decrease in abundance and diversity of crustaceans has been reported near Mediterranean sea bream and sea bass farms (La Rosa *et al.*, 2001; Fernandez-Gonzalez & Sanchez-Jerez, 2011), as well as Scottish salmon farms (Hall-Spencer & Bamber, 2007). However, in the present study some amphipod families showed a positive response to the moderate impact from the fish farm, substantially increasing their abundances under these conditions. We detected an increase in the Nuuanuidae family, which commonly shows high population densities in response to organic enrichment or build-up of detritus (Carvalho *et al.*, 2007). Therefore, using a multivariate approach comparing affected and control locations, we obtain information on the overall changes in polychaete or amphipod assemblages. This is despite geographical variability in the sensitivity of single family changes, which can be easily interpreted by farm managers. Other authors also propose the use of non-metric multivariate approaches for monitoring aquatic environments. However, they recommend the combination of several taxonomic groups, since this is feasible with the same sampling effort and independent of the scale of research and the occurrence of certain indicator taxa (Mueller *et al.*, 2014). Furthermore, we have already discussed the univariate information provided by some benthic biotic indicators vs. the multivariate information from the whole assemblage data set. The application of benthic biotic indices such as AMBI, BENTIX, BQI, MEDOCC (Birk *et al.*, 2012) is an expensive time-consuming task requiring well-practised taxonomists to identify all the fauna to species level. Consequently, some authors agree that the multivariate approach is actually more appropriate for detecting the influence of aquaculture on the benthic environment (Quintino *et al.*, 2012; Aguado-Giménez *et al.*, 2015).

To reduce the taxonomic effort, family level identification has been extensively employed in monitoring programs and ecological studies (Warwick, 1988; Karakassis & Hatziyanni, 2000; Gomez-Gesteira *et al*., 2003). Theoretically the use of lower taxonomic units such as species or genera provides access to most of the appropriate and accurate autecological information usable in assessing ecological conditions. But this approach is normally time-consuming and larger hierarchal taxonomic groupings (e.g. families, orders) may possess similar ecological traits and redundant ecological functions. This suggests that new information would not be provided by further taxonomic breakdown (Bouchard *et al*., 2005). It appears that assessing benthic changes at family level provides the same results as at species level, without substantial loss of information, and at a more favorable cost/benefit ratio.

In the study area, TN, TS and d ^{13}C were the environmental variables best related to changes in amphipod assemblages, and TP those in polychaete assemblages. In such a way, the important role of some local environmental variables for macrofauna is highlighted using this approach. Nevertheless, for a comprehensive understanding of the environmental relationship between tuna farming and faunal changes, further studies are needed on a regional scale, taking into account multispatial scales such as those used by Fernandez-Gonzalez *et al*. (2013) for seabream and seabass farming.

Heterogeneity of soft bottoms on different spatial scales is common for biotic and abiotic variables, being greater in stressed environments due to aquaculture, even on a small spatial scale (Fernandez-Gonzalez *et al*., 2013). This wide variability should be considered when deciding the correct management measures following the results of a monitoring program, taking into account replication on several spatial scales, using a hierarchical or nested design. Natural variation in sediment properties on small spatial scales may be due to foraging and bioturbating fauna or changes in habitat structure. Some examples are the presence of mäerl, patches of *Caulerpa* spp., seagrass, and changes in granulometry due to illegal trawling. Such factors alter macrofauna assemblages at a small-scale local level (Chapman *et al*., 1995). Clearly, more studies on the influence of aquaculture on benthic habitats that include a hierarchical design incorporating several spatial scales would render a more robust environmental assessment (Fernandez-Gonzalez *et al*., 2013, Mangion *et al*., 2014). Unfortunately some monitoring programs using biotic indexes are still in fact carried out by analyzing only a single replicate.

Conclusions

Considering that the mandatory survey is an additional financial burden for fish farmers, it would be desirable for the proposed monitoring programs to be well balanced between sampling design and taxonomical effort. We argue that comparing changes in macrobenthic assemblages using a multivariate approach and surrogating the whole benthic assemblage to a single taxocene, such as polychaetes or amphipods identified at family level (Ellis, 1985), is the optimal approach from a cost/benefit point of view. This has been proposed by other authors (Aguado *et al.*, 2015), since sensitive taxa can be used as surrogates able to reflect the patterns of the whole macrobenthic community along both natural and human-induced gradients (Olsgard *et al.*, 2003; Olsgard & Somerfield, 2000; Wlodarska-Kowalczuk & Kedra, 2007). Experimental design should be robust enough to detect the effect of tuna farming over a background of natural heterogeneity and other human impacts. This includes the use of control locations and several scales of spatial replication, avoiding pseudoreplication (Hurlbert, 1984). We suggest the impact of tuna farming be monitored by i) comparing the changes in macrobenthic assemblages, such as amphipods and polychaetes, with the whole benthic assemblage surrogated to one or two taxocenes, ii) using several control locations with a hierarchical design and iii) applying statistics based on a resemblance matrix from a multivariate perspective.

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