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Biofilm responses to marine fish farm wastes

Short title: Biofilm responses to fish farming

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ABSTRACT

The structural, trophic and element accumulation changes in the biofilm community due to organic matter enrichment, eutrophication and metal contamination derived from fish farming were studied. The biofilm biomass, polysaccharide content, trophic niche and element accumulation was quantified along an environmental gradient of fish farm wastes in two seasons. Biofilm structure and trophic diversity was influenced by seasonality as well as by the fish farm waste load. Fish farming enhanced the accumulation of organic carbon, nutrients, selenium and metals by the biofilm community. The accumulation pattern of these elements was similar regardless of the structure and trophic niche of the community. This suggests that the biofilm communities can be considered a reliable tool for assessing dissolved aquaculture wastes. Due to the ubiquity of biofilms and its wide range of consumers, its role as a sink of dissolved wastes may have important implications for the transfer of aquaculture wastes to higher trophic levels in coastal systems.

Keywords: metal accumulation, aquaculture dissolved wastes, organic matter enrichment, community trophic niche, biofilm, periphyton.

Capsule: Under the influence of fish farming biofilm accumulates organic carbon, nutrients, selenium and metals, regardless of the structure and trophic niche of the community.

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INTRODUCTION

Biofilms are ubiquitous communities since it invariably develops on all solid surfaces exposed to aquatic environments (Allison and Gilbert, 1992; Rao et al., 1997), where it represents most of the natural microbial population (Costerton et al., 1995). Biofilms are aggregates of heterogeneous organisms that are attached to each other and/or to a surface. This community is principally constituted by bacteria and microalgae which secrete an extracellular polymeric substance matrix, mainly formed of polysaccharides, which facilitates the attachment of the community to any surface (Characklis and Marshall, 1990). The dissolved fraction of the organic carbon constitutes the main source of energy for bacteria and algae in biofilms (Lock and Ford, 1985), and they also assimilate nutrients and metals in their dissolved form (Das et al., 2009). The exopolymers of biofilms, due to their physical nature, have a great adsorptive capacity and so a great binding affinity for nutrients and metals (Quigley et al., 2002; van Dam et al., 2002; Aldridge et al., 2010), a characteristic that confers biofilms an important accumulation capacity.

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Moreover, biofilm communities are the food source of many types of organisms in aquatic systems such as, invertebrates, including rasping grazers, deposit, planktonic and subdeposit feeders, fish and higher vertebrates (Baird and Thistle, 1986; Decho and Moriarty, 1990; Abreu et al., 2007; Kuwae et al., 2008). Thus, biofilms are considered to represent a trophic link between dissolved compounds in the water column and the higher trophic levels of the ecosystem (Hynes, 1970).

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In addition, biofilms can contribute substantially to energy flow and nutrient cycling (Battin et al., 2003), especially in the nitrogen cycle (Baldwin et al., 2006), since in

aquatic ecosystems, bacteria play an essential role in mineralization and nutrient cycling (Azam et al., 1994; Azam, 1998).

Marine fin fish farming releases substantial amounts of allochthonous organic matter, nutrients and metals to the environment, an effect that can be noticed up to tens or hundreds of metres (Pitta et al., 1998; Karakassis et al., 2000; Morrisey et al., 2000; Pusceddu et al., 2007; Dean et al., 2007). The impact of fin fish aquaculture has been widely reported in the benthos, where common degradation patterns have been observed (Kalantzi and Karakassis, 2006), since the seabed is able to record possible detrimental effects to the environment over long periods of time (Danovaro, 2003). In the water column however, the effects are less obvious (Neofitou and Klaoudatos, 2008), and any differences in the measured parameters are usually more influenced by seasonality than aquaculture wastes (Pitta et al., 1998; Yucel-Gier et al., 2008). Indeed, water column parameters are often not correlated with the extent of benthic impact. This negligible effect in the pelagic system has been attributed to the important diluting effect of the sea (Pitta et al., 2006), to the rapid grazing of planktonic ciliates (Pitta et al., 2009) or to the importance of heterotrophic over the autotrophic bacteria (Navarro et al., 2008).

Biomonitoring is a more powerful tool for assessing aquatic ecosystem health than physical and chemical analyses (Morin et al., 2008). Biofilm communities has been shown to be sensitive to anthropogenic disturbances such as organic matter enrichment, eutrophication and metal pollution (Vis et al., 1998; Admiraal et al., 1999; Ivorra et al., 1999; Barranguet et al., 2002; Khatoon et al., 2007; Morin et al., 2008). Using these communities on artificial surfaces facilitates the direct comparison between sites without confounding environmental and physical variables (Webster and Negri, 2006).

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The analysis of biofilms enables medium term rather than momentary states of the studied ecosystem (Brummer et al., 2003). Therefore, biofilms could provide the "memory" of disturbance that the water column seems to lack. Fish farming provides an appropriate scenario to study the effects of some of the most common forms of aquatic pollution (organic enrichment, eutrophication and metal pollution) in biofilm communities in open sea environments.

In ecological studies, stable isotope analyses have emerged as reliable tools for elucidating the trophic niche and inferring pathways of energy flow in food webs (Cifuentes et al., 1988). This method involves the comparison of stable isotope ratios between consumers and food supplies (Deegan and Garritt, 1997). While $\delta^{13}C$ allows the carbon source to be differentiated, $\delta^{15}N$ permits the relative trophic position of an organism to be assessed. Thus, as in the case of organisms, $\delta^{13}C$ and $\delta^{15}N$ analyses can be applied to whole communities (Kuwae et al., 2008; Marin-Guirao et al., 2008), providing information on changes in the trophic niche of the community. Recently, new metrics have arisen which allow ecologists to quantitatively characterize community-wide aspects, providing new perspectives on food web structure, function and dynamics at a community level (Layman et al., 2007). These metrics, which will be detailed in the Materials and Methods section, have already been applied demonstrating changes in niche variation due to a different number of food sources (Darimont et al., 2009).

The aim of this work was to study the structural, trophic and element accumulation changes in the biofilm community due to organic matter enrichment, eutrophication and metal contamination derived from fish farming. To do this, we measured biofilm biomass, polysaccharide content, trophic niche and element accumulation along an

environmental gradient of fish farm wastes in two seasons with differing waste load intensities.

MATERIALS AND METHODS

Experimental design

The study was conducted in the surroundings of a marine fish farm located in Águilas, SE Spain, (western Mediterranean; 37º 24' 56.2" N, 1º 32' 4.0" W), which produces gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). The fish farm consisted of two groups of 12 fish cages with an annual production of 1000 tonnes.

The field assays were performed using glass slides as the artificial substrate for the biofilm community to attached to them. Glass slides were supported by slide holders. The slide holders, in turn, were maintained 3 m below the water surface by an anchoring system and a buoy. Slides were deployed from a fish cage located at the edge of the fish farm facility along a horizontal transect at 0, 20, 60, 120, 350 and 600 m from the fish cage. At all stations the minimum depth of the seabed was 18 m. Maintaining the same depth at all stations meant that the biofilm community that developed on the glass slides were homogeneously affected by physical factors (e.g. temperature and irradiance) and avoided resuspension episodes. The main source of the organic matter and pollutants

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accumulated by the biofilm community were therefore mainly from the dissolved fraction.

Glass slides were deployed in two seasons (June and September) and in each season for 16 days. These seasons were chosen because of the differing amounts of fish feed used and, so different amounts of waste input. Water temperature at the surface were 19-23 ºC and 23-24 ºC for June and September, respectively. During both surveys the currents had a mean value of 0.05 m s^{-1} and the main direction of the current was NE (Valeport 106 current meter, Valeport Limited, Dartmouth, UK; located in the fish farm next to the fish cage at a depth of 15 m). The horizontal transect, along which the glass slides were deployed, was upstream of the prevailing water-current. For a more detailed description of the current regime and the horizontal transect see Sanz-Lázaro et al. (, 2010). In the water column 3 m below the water surface at the fish cage, total ammonia nitrogen and nitrite annual mean values were <0.06 and 0.007 mg l^{-1}, respectively; while at a site with no influence from the fish farm total ammonia nitrogen and nitrite annual mean values were <0.06 and 0.003 mg l^{-1}, respectively (unpublished data).The average feed supplied to each fish cage was 425 and 689 kg day^{-1} in June and September, respectively. During the September sampling, the slides placed 20 m from the fish farm were not found and so could not be retrieved.

After retrieval of the slides, they were stored frozen at -20ºC, and, before each analysis, the biofilm community was scraped off using clean glass slides. The parameters measured were: dry weight biomass, polysaccharide content, the concentration of stable isotopes ($\delta^{13}C$ and $\delta^{15}N$), total organic carbon (TOC), nutrients [total organic nitrogen

(TON) and total phosphorous (TP)], selenium (Se) and metals (Fe, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr, Tl).

During the first survey water samples were taken at each sampling station and TP, Se, Fe, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr and Tl, were analyzed to compare the accumulation capacity of biofilm compared to the concentration in the water column. Water samples were filtered (0.45 μm GF/C Whatman filter) and stored frozen at -20ºC prior to analysis. The main input of contaminants in the studied aquaculture system is feed pellets, which were analyzed in the same way as biofilm samples.

Biofilm structure

Biofilm structure was analyzed by quatifying the biomass and polysaccharide content. In order to calculate biofilm community biomass, samples were dried at 60ºC until constant weight. Because the extracellular polymeric substances are composed of polysaccharides (Smith and Underwood, 1998; Stal, 2003), the polysaccharide content was measured using the modified phenol-sulfuric acid method (Pacepavicius et al., 1997). Briefly, this method is based on the change of polysaccharides to 5-(hydroxymethyl)-2-furaldehyde (HMF) by means of a strong acid and the subsequent development of colour chromogen between phenol and HMF. Then, the colour absorbance is scanned through spectrophotmetry.

Con formato: Inglés (Reino Unido)

Biofilm trophic niche

For TOC, TON and the stable isotope concentrations of $\delta^{13}C$ and $\delta^{15}N$, samples were previously freeze dried and ground. The carbon and nitrogen isotope ratios of the samples were measured with an elemental analyzer Flash EA1112 (ThermoFinnigan) connected with a mass spectrometer of isotopic relationships Delta^{plus} (ThermoFinnigan).

All the isotopic data are reported in the conventional δ notation as follows:

$$\delta^{13}C \text{ or } \delta^{15}N = (R_{sample} / R_{standar} - 1)\ 1000\ (^{o}/_{oo})$$

where R represents the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio for δ^{13} C and δ^{15} N, respectively. All $\delta^{13}C$ values were reported as the deviation relative to the Vienna Pee Dee Belemnite Limestone Standart (v-PDB). The $\delta^{15}N$ standards were calibrated and results were reported relative to atmospheric nitrogen.

Biofilm elemental analysis

For TP, Se, Fe, Al, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr and Tl analysis, samples were freeze dried and then ground. Afterwards, 0.2 g of sample was weighed and placed in a Teflon reactor. After the addition of 3 ml ultrapure water, 5 ml of concentrated HNO_3 (Merk, Suprapur) and 2 ml of 30% H_2O_2 (Merk, Suprapur), the reactor was maintained in a microwave digester for 20 minutes at a maximum temperature of 210 ºC. Following the acid digestion, the content of each vessel was poured into volumetric flasks and

ultrapure water was added to make up the final volume to 25 ml. Then samples were stored at 4 ºC until quantification. The target elements were determined by an inductively coupled plasma-mass spectrometer (ICP-MS Algilent 7500 ce, with Octopole reaction system). The detection limits of the ICP-MS (calculated as three times the standard deviation of the blanks) were sufficiently low to analyse the sample concentrations. Element recovery was verified using certified reference material (Lagarosiphon major, CRM 60; Community Bureau of Reference, Commission of the European Communities).

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Data analysis

Pearson correlation analysis was performed between the biofilm polysaccharide content of June and September, and between the polysaccharide content and biomass of biofilm in each season independently. If data did not meet parametric assumptions, a Spearman correlation analysis was used.

A two-way ANOVA was performed for the biofilm biomass and polysaccharide, TOC, TON, TP, $\delta^{13}C$ and $\delta^{15}N$ content to detect significant differences between treatments in each factor (season and distance from the fish farm) and the interactions between the two factors. When significant differences were found, the Bonferroni post-hoc test was performed. To compare the trend of the measured parameters along the transect from the fish farm between both seasons, the slope of the regressions was compared by the method described in Zar (, 1984), which is equivalent to an analysis of covariance.

Niche variation in the biofilm community was assessed using stable isotopes under a similar conceptual basis as Bolnick et al. (, 2007) . We used six community-level metrics described by Layman et al. (, 2007), briefly: 1) $\delta^{13}C$ range (CR), which indicates the quantity of basal resources and niche diversification at the base of the food web, 2) $\delta^{15}N$ range (NR), which shows the degree of trophic diversity, 3) total area (TA), which reflects the amount of niche space occupied by a community, 4) mean distance to centroid (CD), which shows the overall degree of trophic diversity, and is specially useful in cases with outlier species, 5) mean nearest neighbour distance (NND), which is a proxy of trophic redundancy and 6) standard deviation of the nearest neighbour distance (SDNND), which indicates the evenness of the distribution of trophic niches in a community. For a description of the calculations of each metric and a more thorough explanation see Layman (, 2007).

In order to integrate the several parameters measured we used multivariate analyses techniques using the program Primer (v. 6) and its complementary statistical package PERMANOVA+ (v. 1). A PERMDISP (Distance-based test for homogeneity of multivariate dispersions) analysis was used to measure the dispersion of $\delta^{13}C$ and $\delta^{15}N$, considered together, in all the stations for both seasons. Before the PERMDISP routine, a resemblance matrix was calculated using Euclidean distances following the recommendations of Clarke and Gorley (, 2006) for environmental samples. The analysis comprised 9999 permutations, using as measures distances to centroid and obtaining p-values from permutations. PERMDISP was run at different levels in the design to clarify dispersion effects following the recommendations of Anderson et al. (, 2008). First PERMDISP was run within each sampling stations individually without

considering seasonality (i.e. combining the two factors, station and season). By doing this, we analysed the homogeneity of multivariate dispersion within sampling stations for both seasons. Then, PERMDISP was run between sampling stations considering each season (taking season as a higher factor). With the latter analysis, we used multivariate dispersion as a test for similarity in trophic diversity between the two seasons.

For each season, stations (samples) were ordinated according to the following variables: TOC, TON, TP, Se, Fe, Al, Mn, Cu, Zn, Cd, Pb, Ni, As, Cr and Tl concentrations using a Principal Component Analysis (PCA) routine. The data had been previously normalized to avoid skewness in the analysis due to different element concentration ranges. The obtained eigenvectors, PC1 and PC2, for each variable were plotted to see the accumulation patterns of the analyzed elements with distance from the fish farm. Then, a resemblance matrix using the normalized data was obtained using Euclidean distances following Clarke and Gorley (, 2006) recommendations for environmental samples. All statistical tests were performed with a significance level of $\alpha = 0.05$.

RESULTS

During the retrieval of the glass slides in all the station for both seasons, no macroscopic grazers were observed, indicating that the possible effect of these organisms on modifying the biofilm community was minimal.

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Biofilm structure

Dry weight biomass of the biofilm community ranged from 0.029 ± 0.004 to 0.061 ± 0.008 g and from 0.032 ± 0.006 to 0.071 ± 0.018 g for June and September samplings, respectively (values expressed like this are always mean ± SE). Biomass was significantly higher in the station at 0 m compared with the station at 600 m from the fish farm only in September. The trend of the biofilm community biomass with distance differed significantly between both seasons (Table 1). In the June sampling, biomass decreased with distance from the fish farm up to 120 m and then increased, while in September the biomass showed a continuous decrease with distance from the fish farm (Fig. 1 A).

The polysaccharide content of the biofilm community was much higher in June, when it ranged from 0.26 ± 0.01 to 0.42 ± 0.02 % than in September (0.08 ± 0.02 to 0.13 ± 0.02 %). The polysaccharide content was significantly higher at 0 than at 600 m in June, while there were no significant differences between the same stations in September. In both seasons the polysaccharide content showed a tendency to decrease with distance form the fish farm. The differences were more marked in June (Fig. 1 B), although not to a statistically significant extent (Table 1).

The biofilm polysaccharide content in both seasons (June vs September) was not correlated (R^2 = 0.787 , n = 16 , p = 0.113, Fig. 1 A); nor was the polysaccharide content and biofilm biomass (polysaccharide vs biomass) for either season (June: R^2 = 0.020, n = 18 , p = 0.803; September: R^2 = 0.04 , n = 12 , p = 0.917, Fig. 1).

Biofilm trophic niche

The isotopic signatures clearly differentiated the biofilm community from the fish feed (Fig. 2 A). In fish feed, $\delta^{13}C$, was -22.2 ± 0.06 ‰, the lowest value of all the samples, and showed a significantly different accumulation in the biofilm community during both seasons (Fig. 2 B, Table 1). $\delta^{13}C$ variation in the biofilm community ranged from -20.6 to -17.9 ‰, and from -18.9 to -17.3 ‰ for June and September, respectively (Fig. 2 A). The $\delta^{13}C$ content was significantly lower at 0 than at 600 m from the fish farm in both seasons. Similarly, there were significant differences between the stations placed 0 m from the fish farm in the two seasons, but not between the sampling stations at 600 m (Table 1).

In fish feed, $\delta^{15}N$ was 5.4 ± 0.04 ‰ which was the highest value recorded of all the samples. In the biofilm community, it varied little between sampling stations being in most cases close to 5 ‰, except in the station 0 m from the fish farm in June, when the values were markedly lower (3.3 ± 0.1 ‰). Thus, the trend in $\delta^{15}N$ accumulation with distance was significantly different between both seasons (Table 1). The variation in $\delta^{15}N$ was between 3.1 and 5.1 ‰ and between 4.3 and 5.0‰ for June and September, respectively (Fig. 2 A). The $\delta^{15}N$ content was significantly lower at 0 than at 600 m from the fish farm in both seasons. There were also significant differences between the stations placed at 0 m from the fish farm in both seasons, but no significant differences between the sampling stations located at 600 m in the two seasons (Table 1).

In the biofilm community, the $\delta^{13}C$ range (CR) and, especially, the $\delta^{15}N$ range (NR) for the biofilm community was much wider in June than in September. According to the total area (TA), the trophic niche of the biofilm community was four times greater in June than in September. The mean distance to the centroid (CD) was greater in September than in June, while the mean nearest neighbour distance (NND) and the standard deviation of the nearest neighbour distance (SDNND) showed similar values: 0.179 and 0.128, and 0.186 and 0.103, for June and September, respectively (Table 2). Data for $\delta^{13}C$ and $\delta^{15}N$ indicated that the changes in the trophic niche of the biofilm community were influenced by fish farming as well as by seasonality.

PERMISP analysis of all the sampling stations regardless of the season indicated that the homogeneity of multivariate dispersion was not significantly different within stations ($p=0.794$), while the same routine between the sampling stations as regards season indicated that multivariate dispersion was significantly higher in June than in September.

Biofilm elemental analysis

With the purpose of studying element accumulation in biofilm, all the elements measured were analyzed using a PCA routine. PC1 explained 51 % of the variation and grouped TOC, TON, TP, Cu, Cd, Se and Zn on the one hand and the rest of the elements on the other hand. PC2 explained 27 % of the variation and gathered Fe and Zn in one group and the rest of the elements in another. Taking into consideration both axes of the PCA, TOC, TON, Cu, TP, Se and Cd gathered together, while Pb, Tl, Ni, Cr,

Al, Mn and As formed another group. Fe and Zn were considerably distant from the rest of the elements (Fig. 3).

The TOC content of the biofilm community ranged from 13.4 ± 0.2 to 23.2 ± 0.6 % and from 14.2 ± 0.2 to 24.3 ± 1.2 % for June and September, respectively (Fig. 4 A). There was a significant diminution of TOC with increasing distance from the fish farm, but there were no significant differences between sampling times. Neither were there significant differences in TOC concentrations between seasons in the biofilm community placed at 0 m, but there were significant differences for the one placed at 600 m (Table 1).

TON concentration of the biofilm community was between 2.1 ± 0.09 and 3.9 ± 0.08 % and 2.8 ± 0.05 and 5.3 ± 0.29 % for June and September sampling, respectively (Fig. 4 B). As with TOC, there were no significant differences in the trend of the TON content between both seasons. In both seasons there was a significant decrease in TON with distance from the fish farm, although, in this case, the September values were always higher compared with June (Fig. 4 B, Table 1).

The TP content of the biofilm community ranged between 0.11 ± 0.004 and 0.18 ± 0.008 % and between 0.40 ± 0.025 and 0.77 ± 0.017 % for June and September, respectively. The accumulation trend of TP was significantly different along the spatial gradient between both seasons. There was a significant decrease in the TP content between the sampling stations placed at 0 and 600m from the fish farm in both seasons, although the values were much higher in September and so the decrease between the stations furthest from each other were more marked (Fig. 4 C, Table 1).

The POC, PON and TP ratios in the fish feed were similar to those found in biofilm in both seasons. Of all the elements measured in the fish feed, TOC was the most abundant while Tl was the least abundant. The most abundant metal was Fe, followed by Zn, Al, Mn, Cu and As. The rest of the elements had a concentration below 1 μg g^{-1} (Table 3).

A comparative water analysis showed that only TP, Cu, As Cr, Mn, Ni, and Tl were above the detection limits, ranging between 51.3 and 73.7, 0 and 2.5, 2.9 and 3.6, 1.0 and 1.5, 0 and 0.5, 0 and 0.9, 0.01 and 0.05, μg L^{-1}, respectively. In most of the cases, the concentrations were at least four magnitude orders lower than the concentrations in biofilm. Of these elements, only TP, Mn and Tl concentrations in the water column showed to some extent a decreasing trend with increasing distance from the fish farm.

DISCUSSION

According to the biofilm structure, the biofilm biomass showed quite constant values along the environmental gradient from the fish farm in both seasons. Only the station at 0 m in September showed a higher value, being significantly greater than the station located at 600 m in the same time period (Fig. 1 A). This suggests that biomass only responds significantly at high organic matter loads, which agrees with previous works that shows than eutrophication enhances biofilm biomass (Dodds et al., 2000 and references therein).

The polysaccharide content, as an indirect measure of the extracellular polymeric substances, seemed to be quite constant with distance form the fish farm, indicating that the polysaccharide content was little influenced by the fish farm load (Fig. 1 B). However, the polysaccharide content of biofilm showed a seasonal behaviour and greater values were observed in June than in September. This could be due to the high variability in the composition, structure and amount of the extracellular polymeric substances in different microorganisms that produce it (Fig 1 B Tago and Aida, 1977; Decho, 1994). The seasonally different species composition of the biofilm community may have led to different polysaccharide contents (Riedel et al., 2007; Yucel-Gier et al., 2008).

The analysis $\delta^{13}C$ and $\delta^{15}N$ clearly separated fish feed from the biofilm community samples (Fig. 2 A). The biofilm isotopic C and N signature was within as similar range to that recorded in other studies (Kuwae et al., 2008; Marin-Guirao et al., 2008). Fish feed showed the lowest $\delta^{13}C$ values, indicating a more terrestial source, which could be due to the terrestrial components of the fish feed. In both seasons, the $\delta^{13}C$ signature in the biofilm community was more influenced by fish feed in the stations at 0 m than at 600 m, causing the $\delta^{13}C$ content to increase with distance from the fish farm (Fig 2 B).

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Biofilms are complex communities composed of autotrophic and heterotrophic organisms with different trophic levels. Thus, the $\delta^{15}N$ content of biofilms is an average of the trophic levels of the predominant organisms in biomass. In the present study, the $\delta^{15}N$ content was similar for all the samples except that at 0 m in June, which was markedly lower (Fig. 2 A). The lower $\delta^{15}N$ content in the station at 0 m in June could be due to the presence of more autotrophic organisms than in the rest of the stations.

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The biofilm communities mainly assimilates dissolved elements (Lock and Ford, 1985) and the similar values between fish feed and biofilm as regards $\delta^{15}N$ confirmed that the biofilm community does not directly assimilate fish feed in its particulate and untransformed form.

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As regards isotopic metrics, the total area (TA) and the mean distance to the centroid (CD) were notably greater in June than in September, indicating that the trophic diversity of the biofilm community along the spatial transect was higher in June than in September. PERMISP analysis of all the sampling stations, regardless of the season, indicated that the homogeneity of multivariate dispersion was not significantly different within stations, but was significantly different when the season was considered as a factor. This fact indicated that the variability of the trophic diversity was consistent within stations but was significantly different between stations of the different seasons. So the differences in the total area (TA) and the mean distance to centroid (CD), in both seasons, were due to a significantly higher trophic diversity between stations in June than in September, and not to a high dispersion of replicates within each station.

The similar values of the mean nearest neighbour distance (NND) and the standard deviation of the nearest neighbour distance (SDNND) of the biofilm community for both seasons showed that trophic redundancy was comparable, as was the evenness of the distribution of trophic niches in both times of the year (Table 2). According to the results of this study, both season and the fish farm waste load influenced the trophic niche of the biofilm community.

As regards element accumulation by biofilm, the PCA analysis grouped stations following the environmental gradient in both seasons, indicating that the accumulation pattern in biofilm (either positive or negative) was consistent along the fish farm influence for each season. Cu, Zn and Cd seem to be the main metals released to the environment due to fish farm activities (Dean et al., 2007; Basaran et al., 2010). In the present work, according to PCA, Cu, Cd, Se, and, to some extent Zn, seem to have a similar accumulation dynamics as TOC, TON and TP along the distance gradient (Fig. 3). Cu, Zn and Se are micronutrients which can be toxic at high levels, while Cd is a non-essential element that competes for calcium enzymatic locations (Friberg et al., 1979).

TOC, TON and TP content in the biofilm community showed a trend of exponential decay with distance, although the magnitudes were greater in September than in June, in agreement with the high production in September compared to June due to higher sea water temperatures at this time of the year. This difference between both periods was especially marked for TP (Fig. 4). Fish farm inputs of dissolved organic matter and nutrients were clearly reflected in the TOC, TON and TP contents in the biofilm community. According to the results, the effect of dissolved aquaculture wastes could be noted from 0 up to a point of 120-350 m from the fish farm. The element accumulation pattern seemed to follow the same trend in both seasons, and was consistent with the organic matter load, the accumulation in the biofilm community being higher in September, when the fish farm waste load was higher.

Studying natural biofilms can be problematic, especially when the investigation requires measuring biofilms at a variety of sites. Artificial substrates made of the same material

allow us to increase the reproducibility between sites and minimize confounding influences. Similarly, as biofilms are freshly grown, the results are not confounded by different ages of the biofilm community at the different sites, nor difference in antecedent conditions (Baldwin et al., 2006).

The studied biofilm community showed a high capacity to concentrate elements from the water column released from fish farm activity. This may be attributed to the extracellular polymeric substances and the components of biofilm, which, due to their physical nature, have great adsorptive capabilities (Decho, 1990). According to the results of the present study, element accumulation by biofilm was not correlated with the polysaccharide content of the community, suggesting that the capacity to concentrate these elements was independent of the amount of the extracellular polymeric substances.

This work also shows that biofilm communities can account for the capacity "to memorize" a disturbance effect, which the pelagic community lacks (Brummer et al., 2003). Furthermore, even though both, season and the fish farm waste load, influenced the biomass, polysaccharide content and the trophic niche of the biofilm community, the element accumulation pattern seemed to follow the same trend in both seasons being the accumulation consistent with the fish farm waste load. Hence, the biofilm communities can be considered a reliable tool for assessing the reach and extent of dissolved aquaculture wastes. Due to the ubiquity of biofilms, its adsorptive capacity and its wide range of consumers, the role of biofilms as a sink of aquaculture dissolved wastes may have important implications for the transfer of these wastes to higher trophic levels in coastal systems.

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CONCLUSIONS

This work demonstrates that the biofilm communities are sensitive to fish farm influence, and undergo structural and trophic changes in response. Biofilm structure and trophic diversity was influenced by seasonality as well as by the fish farm waste load. Fish farming enhanced the accumulation of TOC, TON, TP, Se and metals by the biofilm community. The accumulation pattern of these elements was similar regardless of the structure and trophic niche of the community. This suggests that the biofilm communities can be considered a reliable tool for assessing dissolved aquaculture wastes and may have important implications for the transfer of aquaculture wastes to higher trophic levels in coastal systems.

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FIGURE CAPTIONS

Fig. 1. A) Dry weight biomass and B) polysaccharide content (mean ± SE; n=4) in the biofilm community in June (○) and September (●) along the spatial gradient from the fish farm.

Fig. 2. A) $\delta^{13}C$ and $\delta^{15}N$ in the fish feed (×) and the biofilm community at June (empty symbols) and at September (solid symbols) at 0 (○), 20 (▽), 60 (△), 120 (□), 350 (◇) and 600 m (☆) from the studied fish farm. Lines show the isotopic niche widths of the biofilm community in all the sampling stations for June (dashed line) and September (solid line) as total area. B) $\delta^{13}C$ in the fish feed (×) (n=3, mean ± SE) and the biofilm community (n=4, mean ± SE) in June (○) and September (●) along the spatial gradient from the fish farm.

Fig. 3. Principal Component Analysis (PCA) ordination plot of PC1 and PC2 based on the concentration of the analyzed elements in the biofilm community in June and September.

Fig. 4. A) Total organic carbon (TOC), B) total organic nitrogen (TON) and C) total phosphorous (TP) concentration (n=4, mean ± SE) in the biofilm community in June (○) and September (●) along the spatial gradient from the fish farm.

TABLES

Table 1. Results of the two-way ANOVA and analysis of the slope (June vs September sampling) of the regressions of dry weight biomass, polysaccharide content, total organic carbon, total organic nitrogen, total phosphorus, $\delta^{13}C$ and $\delta^{15}N$ of the biofilm community along the spatial transect from the fish farm in June and September. All values are expressed as P values.

Parameter	ANOVA (main test)			ANOVA (pair wise test)				Slope differences (June vs September)
	Distance	Season	Interaction	0 vs 0 m	600 vs 600 m	0 vs 600 m in June	0 vs 600 m in September	
Biomass	< 0.05	n.s.	n.s.	n.s.	n.s.	n.s.	< 0.05	< 0.01
Polysaccharide	< 0.0001	< 0.0001	<0.001	< 0.001	<0.001	< 0.001	n.s.	n.s.
TOC	< 0.0001	< 0.0001	< 0.0001	ns	< 0.05	< 0.001	< 0.001	n.s.
TON	< 0.0001	< 0.0001	< 0.0001	< 0.001	<0.001	< 0.001	< 0.001	n.s.
TP	< 0.0001	< 0.0001	< 0.0001	< 0.001	<0.001	< 0.05	< 0.001	< 0.0001
$\delta^{13}C$	< 0.0001	< 0.0001	< 0.0001	< 0.001	n.s.	< 0.001	< 0.001	< 0.05
$\delta^{15}N$	< 0.0001	n.s.	< 0.0001	< 0.001	n.s.	< 0.001	< 0.05	< 0.01

n. s. = non-significant

Table 2: Isotopic metrics for feed and the sampling stations in June and September. $\delta^{13}C$ range (CR), $\delta^{15}N$ (NR), total area (TA), mean distance to centroid (CD), mean nearest neighbour distance (NND) and standard deviation of the nearest neighbour distance (SDNND).

	CR	NR	TA	CD	NND	SDNND	C centroid	N centroid
June	2.700	2.050	2.810	0.719	0.179	0.128	-19.59	4.55
September	1.621	0.807	0.885	0.460	0.186	0.103	-18.15	4.63

Table 3. Element concentration (μg g^{-1}) in the fish feed supplied to the cultured fish in the studied fish farm. The values are in dry weight (mean ± SE; n=3).

Element	Feed concentration
C	489833 ± 4667
N	68667 ± 1014
P	9447 ± 27.1
Fe	286.9 ± 8.7
Zn	83.71 ± 0.16
Al	57.80 ± 3.63
Mn	29.74 ± 2.66
Cu	8.41 ± 0.06
As	1.63 ± 0.004
Ni	0.94 ± 0.016
Se	0.46 ± 0.006
Cr	0.33 ± 0.018
Cd	0.32 ± 0.007
Pb	0.11 ± 0.008
Tl	0.01 ± 0.0003

Fig. 1.

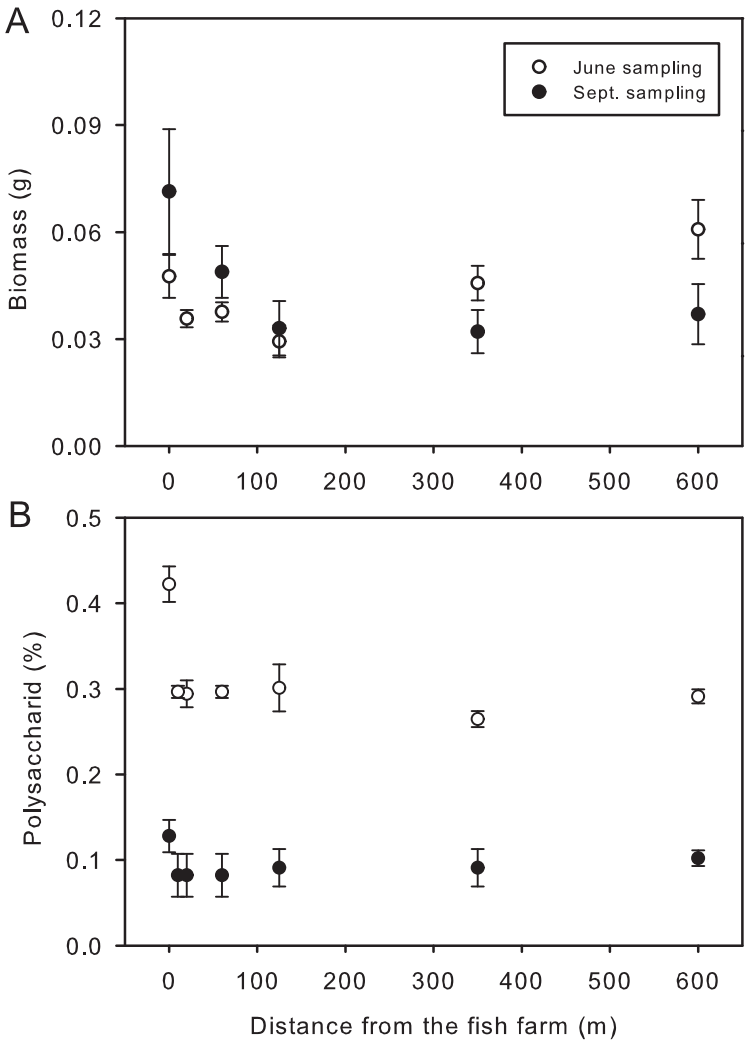

A
B
June sampling
Sept. sampling
Biomass (g)
Polysaccharid (%)
Distance from the fish farm (m)
0.00
0.03
0.06
0.09
0.12
0.0
0.1
0.2
0.3
0.4
0.5
0
100
200
300
400
500
600

Fig. 2.

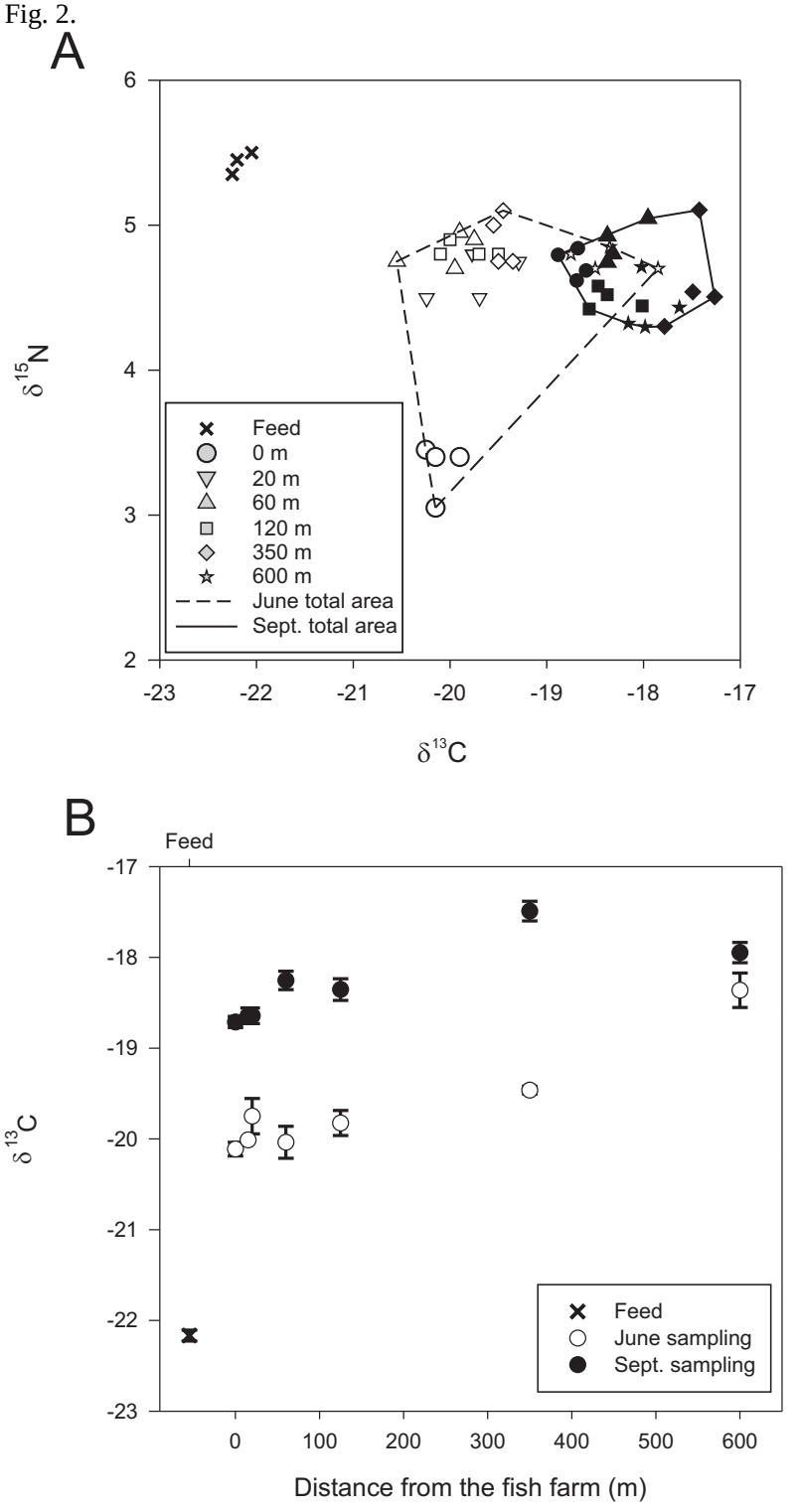

A
6
5
4
3
2
δ15N
-23
-22
-21
-20
-19
-18
-17
δ13C
Feed
0 m
20 m
60 m
120 m
350 m
600 m
June total area
Sept. total area
B
Feed
-17
-18
-19
-20
-21
-22
-23
δ13C
0
100
200
300
400
500
600
Distance from the fish farm (m)
Feed
June sampling
Sept. sampling

Fig. 3.

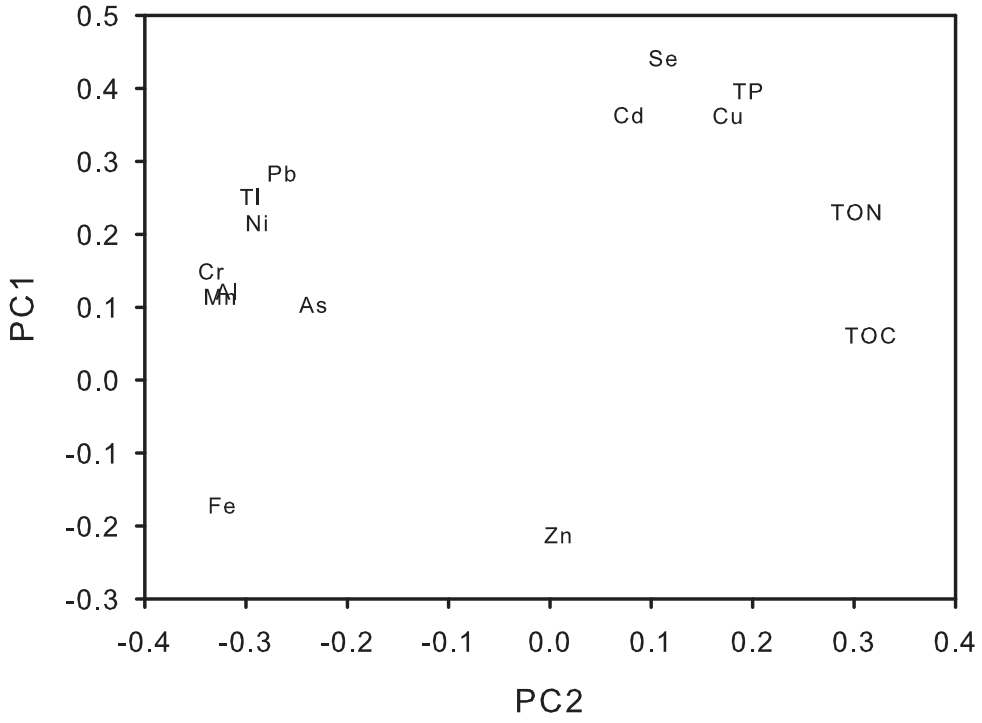
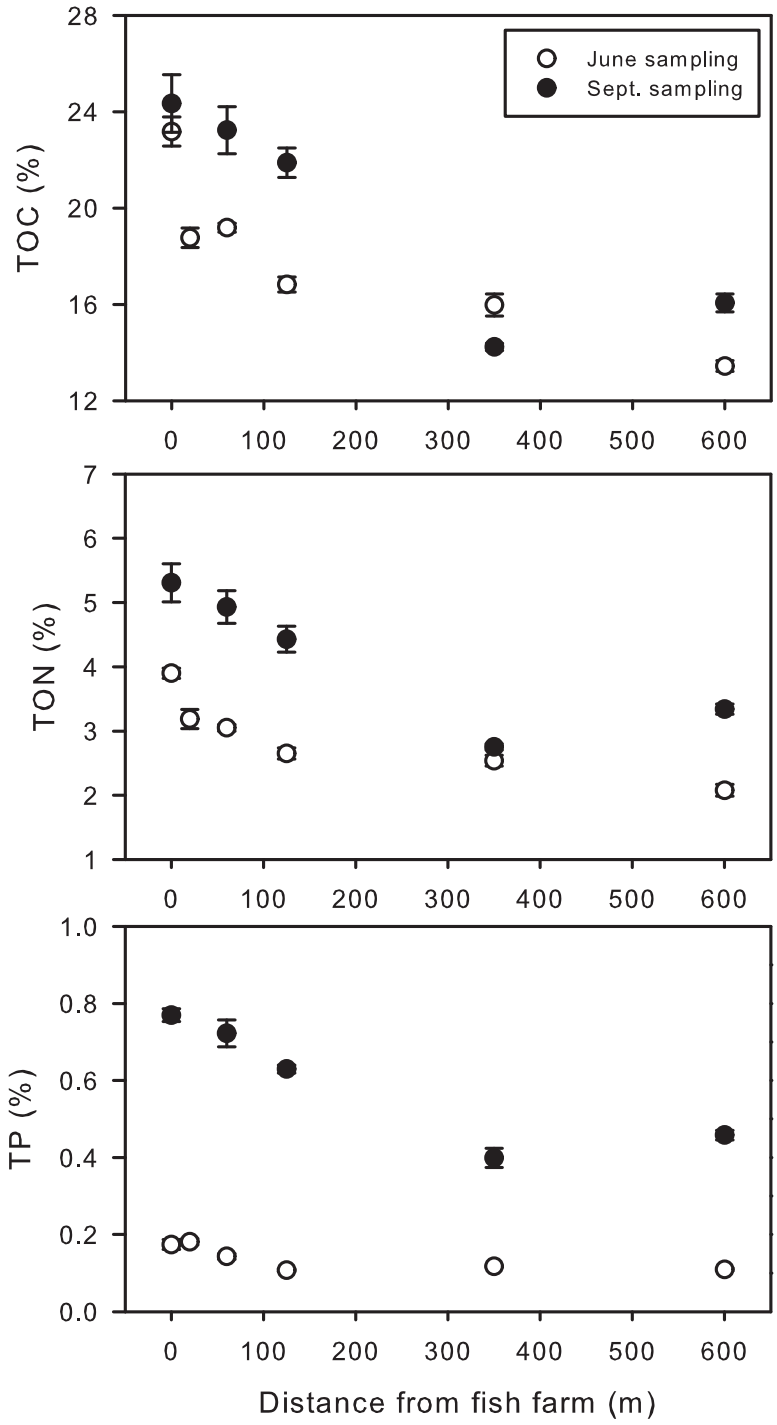

0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
PC1
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
PC2
Se
TP
Cd
Cu
Pb
Tl
Ni
TON
Cr
Al
Mn
As
TOC
Fe
Zn

Fig. 4.


June sampling
Sept. sampling
TOC (%)
TON (%)
TP (%)
Distance from fish farm (m)