Sospedra, J.; Falco, S.; Morata T.; Rodilla, M. (2016). Influence of Organic Enrichment and Spisula subtruncata (da Costa, 1778) on Oxygen and Nutrient Fluxes in Fine Sand Sediments. Estuaries and Coasts. doi:10.1007/s12237-016-0174-1.

Estuaries and Coasts
(2016)
Doi: 10.1007/s12237-016-0174-1

Influence of organic enrichment and *Spisula subtruncata* (da Costa, 1778) on oxygen and nutrient fluxes in fine sand sediments

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Abstract

The role of labile organic material and macrofaunal activity in benthic respiration and nutrient regeneration have been tested in sublittoral fine sand sediments from the Gulf of Valencia (northwestern Mediterranean Sea). Three experimental setups were made using benthic chambers. One experiment was performed *in-situ* through the annual cycle in a well sorted fine sand community. The remaining experiments were carried out with mesocosms under laboratory conditions: one with different concentrations of organic enrichment (mussel meat and concentrated diatoms culture), and the other adding two different densities of the endofaunal bivalve *Spisula subtruncata*. Biochemical variables in surface sediment and changes in oxygen consumption and nutrient fluxes throughout incubation period were studied in each experiment. In the *in-situ* incubations, dissolved oxygen (DO) fluxes showed a strong correlation with sedimentary biopolymeric fraction of organic carbon. Organic enrichment in the laboratory experiments was responsible for increased benthic respiration. However, sediment response (expressed as DO uptake and dissolved inorganic nitrogen –DIN– release) between oligotrophic and eutrophic conditions was more intense than between eutrophic and hypertrophic conditions. *S. subtruncata* abundances close to 400 and 850 ind. m^{-2} also intensified benthic metabolism. DO uptake and DIN production in mesocosms with added fauna were between 60–75% and 65–100% higher than in the control treatment respectively. The results of these three experiments suggest that the macrobenthic community may increase the benthic respiration by roughly a factor of two in these bottoms, where *S. subtruncata* is one of the dominant species. Both organic enrichment and macrobenthic community in general, and *S. subtruncata* in particular, did not seem to have a relevant role in P and Si cycles in these sediments.

Keywords: benthic macrofauna, benthic respiration, nutrient regeneration, biopolymeric carbon, bivalve, well sorted fine sands.

Introduction

Sandy sediments, which cover about 70% of continental shelves (Boudreau et al. 2001), usually contain a relatively small fraction of organic matter and dissolved reactants (Ehrenhauss and Huettel 2004), which suggests that these sediments are hardly active biogeochemically. However, approximately 24–73% of the shelf benthic respiration takes place on permeable sediments (Huettel et al. 2014 and references therein). Benthic respiration, and also nutrient regeneration, are controlled by an important number of factors such as: temperature (Thamdrup et al. 1998), light penetration (Colijn and de Jonge 1984), sediment grain size (Huettel et al. 2014 and references therein),

sediment topography (Røy et al. 2002), wave currents (Jørgensen and Revsbech 1985), microbial community (Danovaro et al. 1999), phytobenthos (Viaroli et al. 1996), benthic meiofauna (Moodley et al. 2008), macrofauna (Lohrer et al. 2004) and organic material (Pastor et al. 2011). Both the macrofauna community and sedimentary organic matter are determinant drivers of benthic metabolism in shallow coastal waters (Mermillod-Blondin and Rosenberg 2006; Pastor et al. 2011). In these areas, 75–90% of suspended particles from river discharges reaches sea floors (Mantoura et al. 1991; Pernetta and Milliman 1995), representing the main energy source for benthic macrofauna (Venturini et al. 2012).

Organic matter that reaches marine bottoms can be of both continental and marine origin and is composed of labile and refractory compounds. Specifically, the labile fraction of sedimentary organics is characterized by high sensitivity to oxidation (Arnosti and Holmer 2003). However, not all organic compounds have a similar preservation potential. As a general rule, water-soluble organic compounds, or organic macromolecules, which are easily hydrolysed to water-soluble monomers, have a low preservation potential. In contrast to this, compounds with a low solubility in water and hydrolysis-resistant macromolecules are selectively enriched in the sedimentary organic matter (Rullkötter 2006). In addition, the labile fraction increases the nutritional quality of sedimentary organic matter (e.g., by protein enrichment and by available food source for macrofauna) (Danovaro and Fabiano 1997). Accumulation of organic compounds is the triggering mechanism leading to changes in physical, chemical, biological and ecological features of sediments (Cloern 2001). Organic matter in sediments is mineralized to inorganic compounds through microbial processes using different electron acceptors (Canfield et al. 1993). Initially, this organic enrichment means a higher uptake and total use of all the oxygen contained in the surface layers of the sediment. This leads to an increase in the use of other oxidants such as sulphate and stimulates sulphide production (Holmer and Kristensen 1994), toxicity and anoxia in sediments (Piedecausa et al. 2012). Organic particle accumulation over the bottoms diminishes sediment stability, increases resuspension and, therefore, increases water column turbidity (Lundkvist et al. 2007; Canal-Verges et al. 2010). An intense organic enrichment can also affect the macrobenthic community, altering it and causing it to be replaced by communities that are more resistant to organic pollution (Pearson and Rosenberg 1978; Hargrave et al. 2008). Consequently, changes in the trophic status can disturb the functioning of the benthic system, implying effects in the whole ecosystem (Venturini et al. 2012). In this sense, some authors have demonstrated that levels of biopolymeric compounds can inform us about organic enrichment in benthic systems (Cotano and Villate 2006; Pusceddu et al. 2009). So, the labile fraction of sedimentary organic matter is used as an organic pollution descriptor to assess the trophic status of the benthic compartment (Dell'Anno et al. 2002). The main biochemical compounds used for this purpose are proteins, carbohydrates and the biopolymeric fraction of sediment organic carbon, measured as the sum of protein,

carbohydrate and lipid carbon (Dell'Anno et al. 2002). However, only a few studies, such as Pastor et al. (2011) and Sospedra et al. (2015), have focused on the relation between oxygen consumption and nutrient cycling by sediments and specific descriptors of the labile fractions of sedimentary organics.

Benthic macrofauna, due to its activity (herbivory, predation, biodeposition and bioturbation) can promote certain biochemical processes at the expense of others, modifying physical, chemical and biological characteristics of sediments (Aller and Aller 1998; Gerino 1990; Gilbert et al. 1995; Kristensen et al. 2012). In this sense, each species of benthic organisms behaves in a different way and therefore has different effects on biochemical processes in sediments. For instance, bioturbator species, such as nereid polychaetes, redistribute sediment particles while burrow-dwelling organisms create an intense exchange of water between sediments and the overlying water column (Kristensen et al. 2012). In general, macrofauna activity increases various nutrient fluxes from sediment to water column (Bartoli et al. 2001; Pratihary et al. 2009). Nevertheless, a high bioturbator density could promote phosphorus precipitation with oxyhydroxide particles (Mortimer et al. 1999), thereby increasing phosphorus concentration in sediments.

Macrobenthic communities inhabiting fine sands in the Mediterranean are composed of small-size polychaetes, crustaceans, echinoderms and molluscs including gastropods, scaphopods and bivalves (Bellan-Santini et al. 1994). In particular, *Spisula subtruncata* (da Costa 1778) is one of the most abundant bivalves in coastal areas of Europe, with a distribution from Norway to Morocco and all around the Mediterranean Sea (Deval and Göktürk 2008). In some areas, *S. subtruncata* represents a major food source for some diving ducks (Baptist and Leopold 2009), demersal fish (Braber and De Groot 1973), shrimp (Pihl and Rosenberg 1984), and even represents an economic resource (Rueda and Smaal 2004). This species inhabits the lower shoreface at water depths of about 5–20 m in fine sands with an average grain size of about 200 μm (Baptist and Leopold 2009; Degraer et al. 2007). In addition, *S. subtruncata* plays a key role in macrobenthic community structure due to its dominance, especially during spring and summer (Fraschetti et al. 1997). It is a filter feeder which lives partially buried within the first 5 cm (Demestre et al. 2007) and has limited movement (Queirós et al. 2013). These two traits involve a lower bioturbation potential than other benthic organisms with free three-dimensional movement via burrow system, such as nereids or corophiums (Queirós et al. 2013). However, its bioturbation activity could become more intense in the uppermost sediment layers due to both particle reworking and defecation at the sediment surface (Dauwe et al. 1998) at high densities. This latter biological process, also called biodeposition, represents an important food source available for detritivores, as well as suspensivores because biodeposits can be easily resuspended (Newell 1979).

The aim of this work is to document the relationship of benthic metabolism and nutrient cycling with both sedimentary organic composition and macrofauna, focusing on *S. subtruncata,* in sublittoral permeable sands. For

this purpose, three different experiments were carried out: *in-situ* incubations and two *ex-situ* studies with mesocosms, one of them inoculating organic matter and the other adding the bivalve *S. subtruncata*. Two levels of organic enrichment and *S. subtruncata* densities, as well as control treatments, were reproduced in each laboratory experiment. This experimental approach allowed us to examine: 1) oxygen and nutrient fluxes response to changes in quality and quantity of organic compounds and macrofauna composition in sublittoral fine sand sediments throughout annual cycle; 2) the benthic metabolism with increasing organic loading; 3) *S. subtruncata* effect on sedimentary organic composition and oxygen and nutrient exchange at the sediment-water interface.

Material and Methods

In-situ experiments

Site description and sampling campaigns

The study site, where *in-situ* sampling campaigns were carried out, was located at the southernmost sector of the Gulf of Valencia (western Mediterranean Sea) on the coastal area of Gandia (Spain). Seawater in this area has been characterized as having oligotrophic conditions and with particulate suspended matter levels generally below 10 mg L^{-1} (Gadea et al. 2013; Sebastiá et al. 2013; Sebastiá and Rodilla 2013). The sampling station (39º 00' 37" N, 00º 09' 19" W) was at a depth of approximately 9 m, where the seabed is composed of 90% fine and very fine sands. These sediments were moderately well sorted with an average grain size value of 0.138 mm (Sospedra et al. 2015) and a porosity of approximately 0.47. The community was composed mainly of polychaetes, molluscs, crustaceans and echinoderms between 0.5–20 mm in size.

Eight different sampling campaigns were carried out over several annual cycles at the study site to take into account seasonal variations that occur in this coastal area. Sampling campaigns took place in Summer 2009 (5th August), Winter 2010 (17th March), Spring 2010 (17th June), Summer 2010 (7th September), Spring 2011 (15th June), Summer 2011 (30th August), Autumn 2011 (7th December) and Winter 2012 (15th March).

In each sampling campaign, temperature and dissolved oxygen (DO) were measured with an YSI proODO probe (Yellow Springs, Ohio, USA) at the bottom water layer. Likewise, salinity was determined using a multiparameter probe (Multi 340i WTW, Germany). Twelve undisturbed sediment cores were carefully collected by scuba divers using hand cores (30-cm length, 6.5-cm internal diameter) at the sampling station. Six cores were used for

determining proteins, carbohydrates and lipids in the uppermost 1 cm sediment layer. These cores were kept in the dark and immediately transported to the laboratory. The remaining six cores were used for the study of benthic macrofauna. These were sieved individually using a 500-μm mesh and fixed in 5–10% buffered formaldehyde (Castelli et al. 2004).

Benthic chamber experiments

In-situ benthic incubations were conducted at the same time that the bottom water layer and sediment were sampled. These incubations were used to determine DO, ammonium (NH_4^+), nitrite plus nitrate (NO_2^-+NO_3^-), phosphate (PO_4^{3-}) and silicic acid ($Si(OH)_4$) fluxes between the sediment and water column in each sampling campaign. Three semi-spherical dark chambers of 40 cm internal diameter, previously used in works in the Gulf of Valencia (Morata et al. 2012; Morata et al. 2014; Sospedra et al. 2015), were placed in the sediment in each sampling campaign. Each chamber had a volume of 16.7 L and covered a sediment surface area of 0.125 m^2. Incubations were conducted, between 10–13 hours. During this time, a 180 mL aliquot of incubated water was withdrawn, after homogenization of the water by stirring manually, from each chamber every 2–2.5 hours. Incubated water samples were delivered carefully to the laboratory in less than one hour after their collection.

Laboratory experiments

Two more experiments were carried out with mesocosms under laboratory conditions, one with different concentrations of organic enrichment and the other adding two different densities of the bivalve *S. subtruncata* (Fig. 1). For each laboratory experiment, nine mesocosms (cylinders of 20-cm diameter x 30-cm length) were distributed in three treatments (three cylinders per treatment).

Collection of sediment and experimental setup

Sediment, used in both laboratory experiments under regulated conditions, was collected from the *in-situ* sampling station. Thus, identical physicochemical conditions to those found in the study area were guaranteed. Sediment was sieved (1-mm mesh) to remove macrofauna and larger particles and then frozen at -20 ºC until experiments were implemented (during at least two weeks) to kill infauna and most of the microbes (azoic sediment) (Emmerson et al. 2001; Solan et al. 2008).

The experiments were carried out in environmental chambers in which temperature was kept controlled at 19 ºC, which is the average temperature found in coastal areas in the Gulf of Valencia (Mayer et al. 2012). During acclimation, the chambers were maintained under a regulated photoperiod (12h light/12h dark) supplied by four fluorescent tube lights at 2000 lux. The nine chambers were filled to 12 cm in height with the azoic sediment previously defrosted. In addition, they were filled with 6 L seawater from the sampling station that was aerated and renewed (mean water renewal: every 8 h) during the acclimation. In the last 24 h of this stage, the water was replaced by UV-sterilized, 2-µm filtered seawater to allow removal of suspended particles.

Organic enrichment experiment

Three different levels of organic enrichment were tested ($control_{(OE)}$, OE+ and OE++) in triplicate (Fig. 1). Mussel meat (previously dried, ground and 2-mm sieved) plus concentrated diatom culture (*Chaetoceros sp.*, previously frozen with a chlorophyll *a* concentration of 66.5 µg L^{-1}), were chosen as sources of organic matter. This organic matter was selected for its marine origin and lability. In control chambers, only azoic sediment was incubated. For OE+ treatment, 23.5 g of mussel meat and 50 mL of concentrated culture of diatoms were inserted within the uppermost 4 cm of azoic sediment. In the OE++ treatment, 42.5 g of mussel meat and 100 mL of frozen diatoms were supplied. Acclimation lasted four days.

S. subtruncata *experiment*

Different densities of *S. subtruncata* were assembled ($control_{(Ss)}$, Ss+ and Ss++) in this experiment, using three replicates of each one (Fig. 1). *S. subtruncata* was chosen because it is one of the most abundant and representative species in the well sorted fine sands community in the Mediterranean Sea (Sardá et al. 2000; Deval and Göktürk 2008). All individuals used in this experiment were collected from the seabed next to the sampling station. Their length and biomass were 1.37±0.16 cm and 0.37±0.08 g DW $ind.^{-1}$, respectively, expressed as mean±standard deviation (n=30). In the Ss+ treatment, 12 individuals of *S. subtruncata* were added (corresponding to a density of 414 ind. m^{-2}) to each chamber, whereas in the Ss++ 25 individuals were added (863 ind. m^{-2}). Moreover, controls without individuals were carried out (Fig. 1). The chosen densities were similar to those natural abundances for this species in the western Mediterranean (Fraschetti et al. 1997; Sardá et al. 2000). Acclimation lasted 11 days, and consisted of two stages. Firstly, the azoic sediment was allowed to settle and recover from sediment installation for

three days before introducing the bivalves. Then, the organisms were added and kept for eight more days to acclimatize them to laboratory conditions.

Sampling procedures

Following acclimation, incubation was carried out in dark conditions and without seawater renewal for 8–13 hours. During this phase, six incubated water samples were carefully collected at regular intervals to analyse DO and dissolved nutrients (NH_4^+, NO_2^-+NO_3^-, PO_4^{3-} and $Si(OH)_4$). Incubation period and sampling intervals were adjusted so that DO concentrations in incubated water never fell below 70% of their initial value (Pastor et al. 2011). Before collection, incubated seawater was homogenized by stirring manually in order to prevent concentration gradients. The withdrawal of the water sample was compensated by inserting the same volume of filtered seawater in from a vent placed at the top of each chamber. Moreover, when incubation finished, the uppermost centimetre layer from each chamber was collected by small hand cores to determine proteins, carbohydrates and lipids.

Sample analyses

Water samples incubated from *in-situ* benthic chambers and laboratory mesocosms were used to analyse DO and nutrients. The DO samples were fixed immediately and analysed using the Winkler iodometric method (Aminot and Chaussepied, 1983). For the analysis of dissolved nutrients, the samples were filtered using a cellulose acetate filter with a 0.45-µm pore diameter. NH_4^+, NO_2^-+NO_3^-, PO_4^{3-} and $Si(OH)_4$ were determined according to Aminot and Chaussepied (1983). Dissolved inorganic nutrient (DIN) was calculated as the sum of NH_4^+ and NO_2^-+NO_3^-.

Surficial sediment samples were used to analyse the main biochemical compounds: proteins, carbohydrates and lipids according to Pusceddu et al. (2004). These analyses were carried out on six replicates for each biochemical variable measured. These biochemical compounds were converted to carbon equivalents assuming a conversion factor of 0.49, 0.40 and 0.75 µgC μg^{-1} for proteins, carbohydrates and lipids, respectively. The biopolymeric fraction of sediment organic carbon was obtained as the sum of proteins, carbohydrates and lipids carbon equivalents (Fichez 1991).

Flux calculations

Benthic fluxes across the sediment-water interface were obtained by fitting the concentration changes of the chemical species over time to linear expressions in each chamber. Initial (C_i) and final concentrations (C_f) were recalculated from linear fits to determine benthic fluxes expressed in μmol m^{-2} d^{-1}, using the equation: $F=((C_f-C_i)\ V\ 24)/(t\ A)$, where C_f and C_i represent final and initial concentration, respectively (μM); t is the incubation time (h); and V and A are the chamber volume (L) and the surface area (m^2), respectively. In the case of *ex-situ* experiments, the volume of water withdrawal represented 18% of the incubated water. Hence, DO and nutrient concentrations in the replacement water were known in order to correct solute concentrations in incubated water before calculating the fluxes.

Data processing and statistical analysis

For the *in-situ* studies, water column and sedimentary data were grouped by seasons. They were obtained as the average of the different samplings conducted in the same season. For instance, the winter and spring values were obtained as the average of two sampling campaigns, whereas average values in summer were calculated from three campaigns. In autumn, a single value was obtained from the December 2011 campaign.

Regarding macrofauna, MEDOCC (MEDiterranean OCCidental) index was calculated using macrobenthic community data in order to assess ecological status. The results were rescaled and expressed as a standardized measurement (Ecological Quality Ratio, EQR), which ranged from 0 to 1 as bad and high ecological status respectively (Pinedo et al. in GIG 2008). This biotic index is mainly based on the degree of sensitivity/tolerance to organic enrichment for every taxon found within a community, classifying the species in four ecological groups (sensitive, indifferent, tolerant and opportunistic) to calculate the index depending on the relative percentage of these groups.

The Spearman-Rank correlation analysis was performed in order to test relationships between ranked variables both in *in-situ* and *ex-situ* experiments that do not conform to a bivariate normal distribution or are linearly related. Spearman-Rank tests were conducted using SPSS. Kruskal Wallis tests and multiple comparisons of groups with Bonferroni adjustment of *p*-values were implemented to assess statistically significant differences. The factors chosen were season (winter, spring, summer, autumn) for variables analysed in the *in-situ* experiment, and treatment (control, +, ++) for variables determined in both laboratory studies. These non-parametric statistical tests were carried out with R statistical software.

Results

In-situ sampling campaigns

Environmental factors

Water temperature showed the minimum value in winter, whereas maximum values were observed in summer. These seasonal variations represented differences at all levels of significance between the four seasons (Table 1). Salinity ranged slightly from 36.9 to 38.1, showing significant differences between seasons. DO levels reached a value of 9.5 mg L^{-1} during winter campaigns, being significantly higher in this season and autumn than in the remaining campaigns (Table 1).

Proteins varied between 452 and 569 μg g^{-1} in winter and summer respectively, whereas carbohydrates ranged between 330 and 559 μg g^{-1}. Lipids presented higher fluctuations over the annual cycle, representing statistical differences (Table 1). The biopolymeric fraction of sediment organic carbon reached a maximum of 902 μgC g^{-1} in spring and a minimum value in autumn (Table 1). The levels of labile fraction of sedimentary organics analysed in the study site corresponded to oligotrophic values, as they were below 1500 μg g^{-1} of proteins, 5000 μg g^{-1} of carbohydrates and 1000 μg g^{-1} for biopolymeric carbon, according to the criteria established by Dell'Anno et al. (2002) and Pusceddu et al. (2011).

Polychaetes, molluscs and crustaceans dominated the macrobenthic community, although with varying percentages between seasons. During winter, the well sorted fine sands community was dominated by molluscs, especially juvenile individuals of *S. subtruncata*, with a density above 100 000 ind. m^{-2} (Fig. 2A). This season also showed the highest crustacean abundance. In spring, the three dominant taxonomic groups were present in similar proportions. Polychaetes and crustaceans were the most abundant in summer and autumn with more than the 40% of the community. Regarding taxa, the most abundant and recurrent species were the polychaetes *Magelona filiformis, M. minuta, Paradoneis drachi* and lumbrinerids, the crustaceans *Apseudes bacescui, Ampelisca brevicornis, Iphinoe serrata, Atylus massiliensis* and *Siphonocoetes sabatieri*, and the bivalve *S. subtruncata*. The number of taxa – species richness– varied from 27 collected in winter to 32 taxa determined in summer. In spring and autumn, 31 and 30 different taxa were identified, respectively (Fig. 2B). Index MEDOCC-EQR values were always above 0.75 in each season.

Benthic fluxes

DO benthic fluxes were always negative reaching -37675 µmol m^{-2} d^{-1} in spring (Figure 3A). These fluxes showed a negative correlation with the biopolymeric fraction of organic carbon (r=-0.86, p<0.01).

Regarding nitrogenous compounds, sediments behaved as DIN producers to the water column, exceeding 1000 µmol m^{-2} d^{-1} in three seasons. DIN releases in autumn was significantly higher than other seasons whereas in spring was lower according to the Kruskal Wallis test (Fig 3B). NH_4^+ fluxes in autumn were also the highest –around 2000 µmol m^{-2} d^{-1}– whereas NO_2^-+NO_3^- fluxes ranged between 42±60 and 546±312 µmol m^{-2} d^{-1} in spring and autumn, respectively. PO_4^{3-} fluxes were by far the lowest among the solutes studied, these ranged close to zero. No statistical differences between seasons were determined for this nutrient (Figure 3C). In the case of $Si(OH)_4$, the maximum average flux occurred during winter whereas the minimum occurred in autumn. However, $Si(OH)_4$ fluxes did not show statistical differences either (Fig. 3D).

Laboratory experiments

Sediments

In the organic enrichment experiment (OE experiment), the organic compounds studied increased their concentrations in response to organic enrichment (Table 2). As a result of this enrichment, eutrophic and hypertrophic conditions were achieved in OE+ and OE++, respectively, according to the trophic state classification proposed by Dell'Anno et al. (2002) and Pusceddu et al. (2011). Protein levels were 10-fold higher than $control_{(OE)}$ due to adding organic matter to the sediment. Lipids also showed a similar pattern, increasing four and seven-fold compared to the $control_{(OE)}$ for OE+ and OE++ treatments, respectively. Carbohydrate concentrations were less abundant, ranging from 185±5 to 619±70 µg g^{-1} on average for $control_{(OE)}$ and OE++, respectively. All these biochemical compounds exhibited statistical differences between OE++ treatment and $control_{(OE)}$ (Table 2). The levels observed in these compounds also led to a statistically significant increase in the biopolymeric fraction of organic carbon from the $control_{(OE)}$ to the OE++ treatment. The mean concentration was around 900 µgC g^{-1} in $control_{(OE)}$, 4000 µgC g^{-1} in OE+ treatment and two-fold higher in OE++ treatment regarding OE+.

With regard to the experiment with the bivalves (Ss experiment), protein concentrations decreased as the incubated density of molluscs increased from an average of 380±87 µg g^{-1} in $control_{(Ss)}$ to 269±179 µg g^{-1} in Ss++ treatment (Table 2). Carbohydrate concentrations were slightly lower than the other components although a trend between treatments was not detected. On the other hand, lipids increased as the incubated density of bivalves increased reaching a mean of 450±91 µg g^{-1} in Ss++ treatment. Despite observing variations in the proteins and lipids levels

between treatments (Fig. 4), these trends did not represent significant differences (Table 2). The sum of carbon equivalents of these three compounds showed no pattern among treatments, ranging around 500 µg g^{-1}.

Benthic fluxes

In the OE experiment, an uptake of DO occurred in every one of the incubation chambers. This consumption was more pronounced in those enriched sediments where average benthic respiration was -50535±4116 µmol m^{-2} d^{-1} in the OE+ treatment and reached an uptake of 58415±10745 µmol m^{-2} d^{-1} in the OE++ treatment. A trend close to significance (p=0.06) was detected in DO fluxes where DO uptake in both enriched treatments were higher than in the $control_{(OE)}$ (Fig. 5A). Similarly, DIN fluxes between both enriched treatments and $control_{(OE)}$ exhibited a strong tendency towards statistical significance (p=0.05), showing an increasing trend depending on the organic matter added (Fig. 5B). As well as DIN, NH_4^+ fluxes incremented with increasing organic loading, reaching an average release of 34762±4480 µmol m^{-2} d^{-1} in OE++. NO_2^-+NO_3^- benthic fluxes increased from 52±29 µmol m^{-2} d^{-1} in the $control_{(OE)}$ to 361±158 µmol m^{-2} d^{-1} in the OE++ treatment. In the case of PO_4^{3-}, only OE+ chambers showed an average release of this solute from the sediment (Fig. 5C). An uptake of $Si(OH)_4$ in the OE+ treatment was detected, whereas $Si(OH)_4$ fluxes were positive in the OE++ treatment with an average value over 900 µmol m^{-2} d^{-1}. Consequently, $Si(OH)_4$ fluxes were statistically different between OE+ and OE++ treatments (Fig. 5D).

The most reactive organic matter, described as biopolymeric organic carbon, was correlated with DO fluxes in the *ex-situ* OE experiment (proteins, r=-0.75, p<0.05; carbohydrates, r=-0.62, p<0.10; lipids, r=-0.72, p<0.05; biopolymeric fraction of organic carbon, r=-0.73, p<0.05). Moreover, positive correlations were observed between biochemical compounds and DIN fluxes (proteins, r=0.73, p<0.05; carbohydrates, r=0.90, p<0.05; lipids, r=0.88, p<0.05; biopolymeric fraction of organic carbon, r=0.83, p<0.05).

In the Ss experiment, with *S. subtruncata*, DO fluxes showed a trend towards significance (p=0.06) where DO uptake in $control_{(Ss)}$ was statistical lower compared with Ss+ and Ss++ treatments (Fig. 6A). DIN fluxes were statistical different between treatments with a significance level very close to the established threshold (p=0.05), specifically between both Ss+ and Ss++ treatments and the $control_{(Ss)}$ (Fig. 6B). NH_4^+ fluxes increased with bivalve abundance from 2000 to above 4600 µmol m^{-2} d^{-1} in the $control_{(Ss)}$ and the Ss++. Oppositely, lower fluxes of NO_2^- +NO_3^- were observed when *S. subtruncata* abundance was higher, ranging between 46±106 and 187±206 µmol m^{-2} d^{-1}in Ss++ and $control_{(Ss),}$ respectively. In $control_{(Ss)}$ and Ss+ treatments, an uptake of PO_4^{3-} was observed, whereas in the Ss++ treatment a release was produced, although all of these values were close to zero (Fig. 6C). Regarding $Si(OH)_4$ fluxes, statistical differences were detected only between the control(Ss) and the Ss+ treatment for a close

significance level (p=0.06) (Fig. 6D). Although both treatments with the presence of bivalves showed positive fluxes, the control$_{(Ss)}$ exhibited an average uptake of 64 μmol m^{-2} d^{-1}.

Discussion

Influence of organic matter on benthic metabolism

The sediments in the sampling station showed an oligotrophic state, which may result from their permeable character. Under natural conditions, sandy sediments are subjected to the continuous action of currents and waves, and so tend to accumulate little organic matter. In addition to this, some authors suggest that there are high reaction rates and an intense recycling in permeable sediments, even as high as those reported for organic-rich sediments (Huettel et al. 2014, and references therein). Benthic metabolism on permeable sediments may be enhanced by the labile fraction of organic matter, as suggested by the negative correlation observed between DO fluxes and biopolymeric organic carbon in *in-situ* incubations (r=-0.86; p<0.01). Proteins and biopolymeric carbon levels in the OE+ and OE++ treatments were representative of highly disturbed areas showing a eutrophic and a hypertrophic state, respectively. These scenarios could eventually take place in the marine environment as a result of wastewater discharge, fish-farm effluents, algal blooms deposition, or due to hydraulic regime alteration due to shoreline and offshore constructions. In fact, similar –or even higher– levels of proteins, lipids and biopolymeric carbon have been detected both in Mediterranean coastal areas, such as some points along the Apulian coast (Dell'Anno et al. 2002) and the Mar Piccolo of Taranto (De Vittor et al. 2015) and also in the Rio de la Plata estuary (Venturini et al. 2012).

Fig.7 depicts the DO and DIN fluxes against sedimentary biopolymeric carbon jointly for *in-situ* and *ex-situ* OE experiment. In this figure, a negative trend can be observed for DO and a positive one for DIN, with increasing organic loading. In the OE experiment, the increasing trend observed between DO uptake and DIN release with biopolymeric carbon was mainly associated with proteins and lipids in the sediment. The organic enrichment had a direct impact on the benthic metabolism and nitrogen cycle, probably due to stimulation of microbial development and bacterial processes (Heilskov and Holmer 2001; Fabiano et al. 2003). However, sediment response (expressed as DO uptake and DIN release) between oligotrophic (control$_{(OE)}$ and *in-situ* incubations) and eutrophic (OE+ treatment) conditions was higher than between eutrophic and hypertrophic (OE++ treatment) conditions. In Fig. 7, it can be observed that DO uptake and DIN release rates were less intense as the biopolymeric fraction was increasing, especially from approximately 5000 μg g^{-1} of biopolymeric carbon. In the same way, Heilskov et al.

(2006) observed that mineralization rates were stimulated more by organic enrichment in oligotrophic sediments than in eutrophic sediments. Therefore, oligotrophic sediments may be more sensitive to organic enrichment due to carbon and nutrient-limited growth of the microbial community. In this sense, López et al. (1998) and Holmer et al. (2003) found that organic and nutrient enrichment stimulate microbial activity in oligotrophic sediments in the Mediterranean Sea. High levels of organic matter compounds on the seabed (as a result of anthropogenic effluents or local hydrodynamic conditions) reduce oxygen levels in sediments, increase the sulphate reduction and decelerate the decomposition rate of organic matter (Dell'Anno et al. 2002; Smith 2002; Glud 2005).

However, organic enrichment does not seem to affect the release of PO_4^{3-} at the sediment-water interface. Statistical differences between treatments were not observed despite the phosphorus contained in mussel meat (about 240 mgP/100 g WW according to Fuentes et al. 2009). Probably, the limited exchange observed was on account of a quick phosphorus release during the acclimation period due to its facile hydrolytic breaking of bound phosphate groups (Rullkötter, 2006). With regard to Si, the fluxes did not show a trend related to added organic matter despite statistical differences. The use of dead diatoms culture as a biogenic silica source was insufficient.

Influence of macrofauna on benthic metabolism

S. subtruncata shows a highly variable recruitment success, which gives it a strong seasonal and annual fluctuation within the spatial distribution and density, like many other benthic species that have planktonic life stages (Fogarty et al. 1991; Lewis et al. 2001). For example, a successful recruitment can generate densities above 150 000 ind. m^{-2} (Degraer et al. 2007). This was observed in the sampling station in the 2010 winter campaign when a density above 200 000 ind. m^{-2} was recorded. By contrast, in summer and autumn campaigns no individual examples of this species were found, whereas an average density of 1005 ind. m^{-2} was observed in spring. The data presented for the Gulf of Valencia fall within the ranges observed by other authors around the Mediterranean Sea. Fraschetti et al. (1997) noted high density values above 1000 ind. m^{-2} in the Ligurian Sea, whereas Sardá et al. (2000) observed average annual abundances between 700 and 2600 ind. m^{-2} along the Catalan coast.

The activity of *S. subtruncta* during mesocosm incubations could influence DO uptake through organism metabolic respiration and the intensification of mineralization by the microbial community. Moreover, particle reworking and biodeposition create more propitious conditions to bacterial development. Statistical differences between the $control_{(Ss)}$ and both Ss treatments were observed, with a near-to-significant level, despite similar levels of biopolymeric carbon. It was observed that DO uptake increased 60 and 75% compared to the $control_{(Ss)}$ at abundances close to 400 and 850 ind. m^{-2} of this bivalve (Ss+ and Ss++ respectively). Some authors have also

detected this same response to bioturbation in incubations conducted with the bivalves *Macoma balthica* (Karlson et al. 2005) and *Mya arenaria* (Michaud et al. 2005), resulting in community metabolic respiration and metabolism intensification by the microbial community.

At the same time, *S. subtruncata* enhanced DIN release 65 and 100% for Ss+ and Ss++ treatments, respectively, compared to the control$_{(Ss)}$. Nevertheless, the increase observed for DIN between both Ss treatments and the control$_{(Ss)}$, which was likely to be significant, could be due to two processes: the excretion of NH_4^+ by bivalves (Heilskov et al. 2006); and the organic matter degradation by microorganisms that is encouraged by particle reworking and bioirrigation (Lohrer et al. 2004; Laverock et al. 2011). Furthermore, increased disturbance caused by *S. subtruncata* could intensify nitrification-denitrification coupling. It generates deep oxygen penetration within sediment (Mermillod-Blondin and Rosenberg 2006) and increases the nitrification rate. In turn, this would increase denitrification rates in deeper sediment layers, using oxidized nitrogen compounds. DIN benthic fluxes were composed predominantly of NH_4^+, representing more than 90% in the three treatments of the experiment with *S. subtruncata*. NO_2^-+NO_3^- fluxes were only notable in the control$_{(Ss)}$ where these accounted for about 8% of DIN fluxes; whereas, in Ss+ and Ss++ treatments they represented just 4% and 1%, respectively. Compared to *in-situ* incubation data, the experimental values in Ss experiment were lower, as NO_2^-+NO_3^- release was between 21–47% of DIN release at the sampled station. However, the naturally occurring macrobenthic community, as well as the meiofauna and microbial communities inhabited the sediment during *in-situ* incubations.

The increase of both DO uptake and DIN release highlights the influence that *S. subtruncata*, despite their low bioturbation potential, could have in the exchange of solutes at the sediment-water interface in those cases where *S. subtruncata* is dominant. However, this trend could not be validated working with the macrobenthic community set up for *in-situ* incubations. In this sense, some authors (Emmerson et al. 2001; Biles et al. 2003) have observed that naturally or artificially assembled communities do not increase bioturbation rates nor nutrient fluxes more than single-species incubations. This may be due to the existence of negative or opposing interactions between species (Mermillod-Blondin et al. 2005). Also in this study, the *in-situ* incubations were carried out within a wide temperature range and with a greater variation in studied variables. So, the effects of macrofauna on benthic metabolism could have been concealed by other variables.

Organic matter-macrofauna interactions

The relatively rapid response of the macrobenthic community to environmental changes has been used to obtain a biotic indexes that establishes the ecological quality and trophic state (Pearson and Rosenberg 1978; Dauer 1993;

Borja et al. 2000). Similarly, macrofauna influences sedimentary organics through enhanced mineralization (Martinez-Garcia et al. 2015), selective ingestion of organic particles (Raffaelli et al. 1998) and biodeposit (McKindsey et al. 2011). At the same time, both organic material and macrofauna are able to modify physical and chemical properties in sediments (Hooper et al. 2005 and references therein; Lundkvist et al. 2007). However, neither macrobenthic abundance within *in-situ* incubations, nor the presence of *S. subtruncata* in the *ex-situ* experiment (Ss experiment), showed any statistical relationship with biochemical compounds studied, despite the particle reworking, bioirrigation and biodeposition carried out by these organisms. Only the biopolymeric carbon could have played a particular role in the community structure decreasing species richness. A statistical correlation was detected between biopolymeric carbon and species richness ($r=-0.84$, $p<0.01$ for biopolymeric carbon) in the sampling station sediments. An exclusion of sensitive species is expectable when organic levels increase, thereby leading to a reduction in diversity (Clark 2002).

In this sense, the MEDOCC-EQR index indicated that sublittoral sands in the study site were inhabited by pollution-sensitive taxa. Therefore, a high ecological status (MEDOCC-EQR>0.73) was obtained throughout the annual cycle, according to the criteria established by Borja et al. (2000), modified to the western Mediterranean region by Pinedo et al. (in GIG 2008). Of all taxa determined in these sediments, 43% were classified within trophic group I (sensitive to organic enrichment) most of them belonged to the Class Polychaeta. However, a community that has obtained a high ecological status may also have a low diversity and evenness. This happened in a winter season (winter 2010), where *S. subtruncata*, which belongs to ecological group I, dominated the macrobenthic community completely.

On the other hand, *S. subtruncata* could promote different patterns that may have affected the organic matter quality despite the lack of statistical differences. With similar biopolymeric carbon levels in the three treatments, carbon content in proteins and lipids varied between treatments: a decrease in proteins and an increase of lipids was detected as the bivalve abundance was increased (Fig. 4 and Table 2). It was speculated that changes in the organic compounds composition could have been due to mucus production by bivalves. This is a widespread process among bivalves due to its participation in filtration and rejection processes. Beninger and St-Jean (1997) established that these secretions consist of polysaccharide units with associated protein moieties and they are known both as mucopolysaccharides and glycoproteins. Due to the composition of mucus, it is impossible to tell whether these secretions were the cause of the tendency to increase lipids. The highest abundance of *S. subtruncata* seemed to promote lipid enrichment, possibly due to the accumulation of these compounds, which have higher preservation potential as a result of their low water solubility (Rullkötter 2006). Conversely, compounds with a low preservation potential are more easily hydrolysable to water-soluble monomers, both chemically and enzymatically (i.e.,

proteins) (Rullkötter 2006), possibly due to encouraging the microbial degradation of organic matter by bioturbation and biodeposition. Similarly, Carlsson et al. (2010) reported that only 25% of initial biodeposits of *Mytilus edulis* had degraded after five days. Furthermore, 75% of these biodeposits were composed of relatively refractory material, which is accumulated and gradually degraded in sediments.

To determine the oxygen uptake corresponding to the naturally assembled community in the *in-situ* experiments, the *ex-situ* controls from the OE and Ss treatments were used. These control treatments with sediment from the same sampling station were performed without macrofauna and under similar conditions to those observed in the study site over the annual cycle. In fact, both laboratory experiments were run at 19 ºC, close to the average annual temperature of 19.3 ºC in the *in-situ* campaigns. Organic descriptors also showed similar levels between *ex-situ* controls and the study site. Average biopolymeric carbon concentration in laboratory controls was 693 μgC g^{-1}, an amount close to the average biopolymeric organic carbon at the sampling station, 663 μgC g^{-1}. Both laboratory controls showed benthic flux differences, mainly in DO, despite using the same sediment matrix and incubating under similar conditions. These differences could be due to the acclimation period. For the experiment that added organic matter (OE experiment), the acclimation period lasted 4 days, whereas in the experiment that added bivalves (Ss experiment) this phase lasted 11 days. This difference in acclimation time could allow greater sediment colonization by the microbial community transported by seawater. DO fluxes in control mesocosms of both laboratory experiments were averaged to compare the oxygen consumption in sediments without macrofauna with *in-situ* sediments, inhabited by the natural biotic community. The average DO fluxes from the four seasons *in-situ* samplings reached -15844 μmol m^{-2} d^{-1} and the control average flux was -6600 μmol m^{-2} d^{-1}, suggesting the macrobenthic community may increase oxygen uptake by roughly a factor of two in these sublittoral fine sands. However, within this increase associated with macrofauna it is difficult to determine what percentage accounted for direct uptake due to community metabolic respiration, and what percentage can be attributed to indirect microbial enhancement related to bioturbation. Intensified biogeochemical processes, linked to the DO uptake by macrofauna, may be occurring, modifying nutrient regeneration.

Conclusions

Overall, we have observed how the contribution of organic matter to sediment and the macrofauna community both can stimulate of benthic metabolism in permeable sediments, but in different ways. This type of sediment has a global relevance due to its predominance in coastal zones and its closeness to continental inputs of organic matter. Statistical correlations between DO uptake and DIN release with varying biopolymeric carbon in surficial sediment

suggest that an increase in organic levels stimulates benthic metabolism. However, this stimulation of benthic metabolism was more noticeable in the oligotrophic sediments than in the eutrophic or hypertrophic sediments. *S. subtruncata,* as well as organic enrichment, also intensified benthic respiration and DIN production between 60–75% and 65–100% higher than in the control treatment respectively at the sediment-water interface. Nevertheless, more investigation into the influence that bivalves could exercise on higher preservation potential compounds enrichment and its implications on the benthic metabolism is required. In natural conditions, the macrobenthic community inhabiting this well sorted fine sands community obtained a high ecological status. The macrobenthic community may increase benthic respiration by, roughly, a factor of two in these sublittoral and permeable sediments, where *S. subtruncata* is one of the dominant species. Regarding P and Si biogeochemical cycles, organic enrichment and *S. subtruncata* did not seem to have a significant role in these sediments.

Acknowledgements

This research was supported by the Conselleria d'Educació (Generalitat Valenciana). We are very grateful for the valuable comments of anonymous reviewers on previous version of the manuscript.

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Tables and figures

Table 1

Seasonal averages and standard deviation of variables measured in water column and sediments. The letters following medians indicate the Kruskal Wallis test and multiple comparison of groups with Bonferroni adjustment. For any variable, those seasons that do not share the same letter are significantly different.

ns = non significant

* $p < 0.05$

Treatment		Winter	Spring	Summer	Autumn	α
Environmental factors						
Temperature	ºC	13.0 (2.1)^{a}	21.8 (1.8)^{b}	26.2 (1.0)^{c}	16.4^{d}	*
Salinity		37.5 (0.8)^{a}	36.9 (0.1)^{b}	37.5 (0.2)^{a}	38.1^{c}	*
DO	mg L^{-1}	9.5 (0.4)^{a}	7.5 (0.3)^{b}	7.4 (0.4)^{b}	8.4^{a}	*
Proteins	µg g^{-1}	452 (413)	461 (98)	569 (168)	473	ns
Carbohydrates	µg g^{-1}	559 (203)	363 (54)	398 (160)	330	ns
Lipids	µg g^{-1}	117 (9)^{a}	708 (573)^{b}	259 (12)^{ab}	136^{bc}	*
Biopolymeric carbon	µgC g^{-1}	532 (290)	902 (456)	632 (110)	466	ns

Table 2

Averages and standard deviation of variables measured in sediments at each experiment. The letters following medians indicate the Kruskal Wallis test and multiple comparison of groups with Bonferroni adjustment. For any variable, those seasons that do not share the same letter are significantly different.

ns = non significant

* $p < 0.05$

Sedimentary descriptors	**Organic enrichment**				***S. subtruncata***			
	Control	**OE+**	**OE++**	**α**	**Control**	**Ss+**	**Ss++**	**α**
Proteins (μg g^{-1})	368 [a] (44)	4056 [ab] (801)	6357 [b] (667)	*	380 (87)	308 (61)	269 (179)	ns
Carbohydrates (μg g^{-1})	185 [a] (5)	365 [ab] (24)	619 [b] (70)	*	208 (34)	197 (22)	229 (10)	ns
Lipids (μg g^{-1})	807 [a] (287)	3078 [ab] (525)	6249 [b] (1878)	*	343 (26)	353 (54)	450 (91)	ns
Biopolymeric carbon (μgC g^{-1})	860 [a] (192)	4442 [ab] (421)	8050 [b] (1111)	*	526 (36)	494 (56)	560 (31)	ns

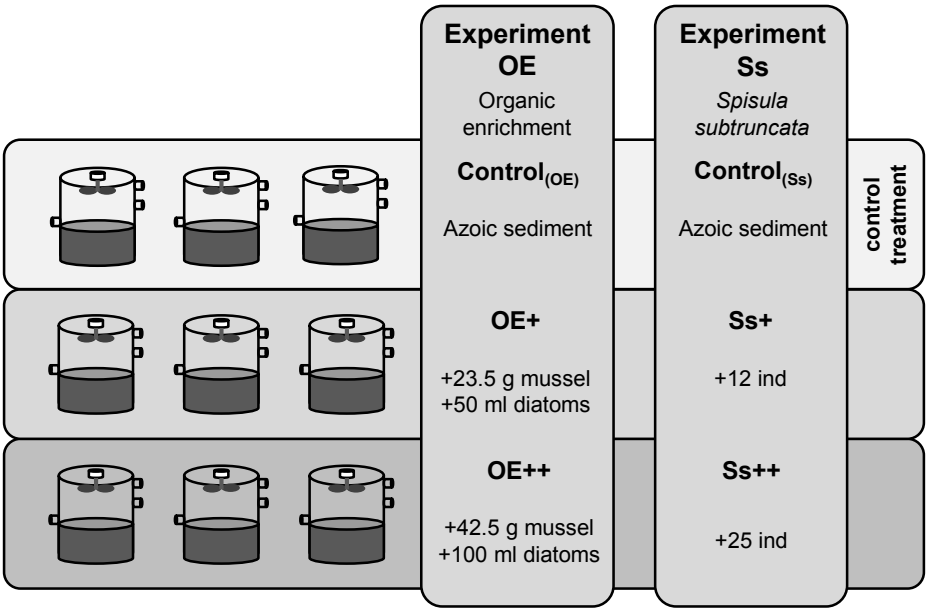

Fig. 1 Outline of *ex-situ* OE experiment with organic enrichment and Ss experiment with *S. subtruncata.*

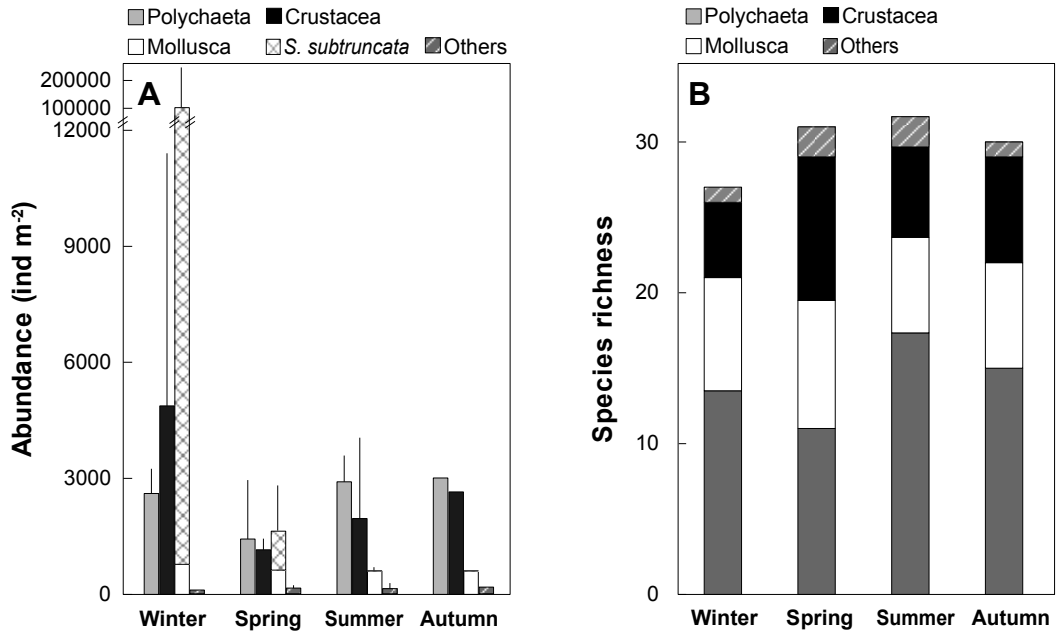

Fig. 2 A Mean abundance of Polychaeta, Mollusca, Crustacea, *S. subtruncata* and other taxa in each season. Error bars represent standard deviation. **B** Species richness of Polychaeta, Mollusca, Crustacea and other taxa in each season.

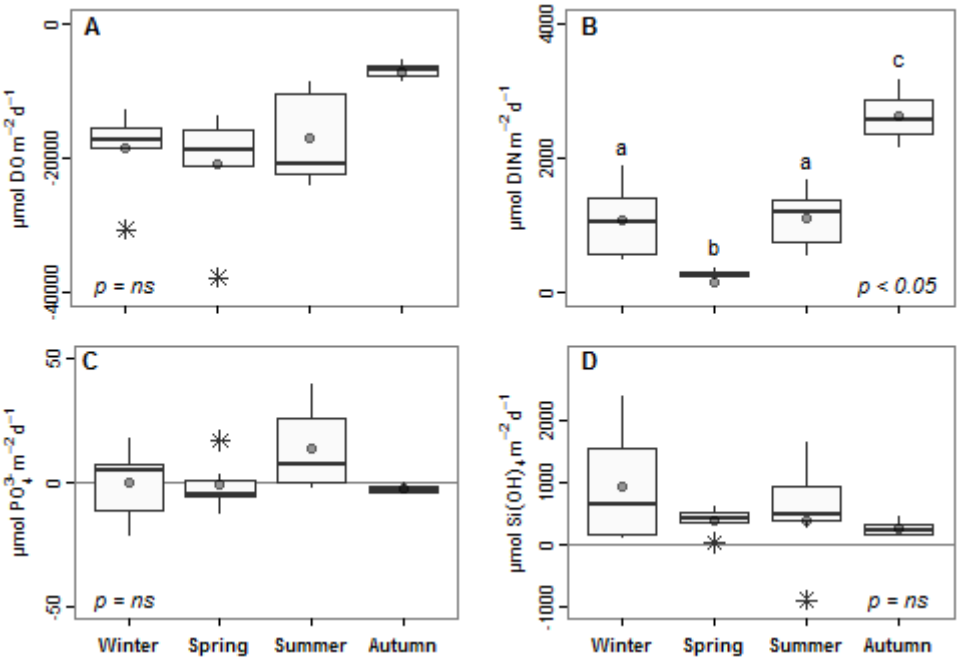

Fig. 3 Box plots of **A** DO, **B** DIN, **C** PO_4^{3-} and **D** $Si(OH)_4$ fluxes at sediment-water interface in the *in-situ* sampling station. The box plots show the inter-quartile range with the median as a horizontal line. Error bars extend from the edge of the box to the highest and lowest values that are within 1.5 * IQR (interquartile range). Asterisks show outliers and grey circles represent mean values. The letters over the box plots indicate the Kruskal Wallis test and multiple comparison of groups with Bonferroni adjustment. Medians with a different letter are significantly different at the significance level indicated on the plot.

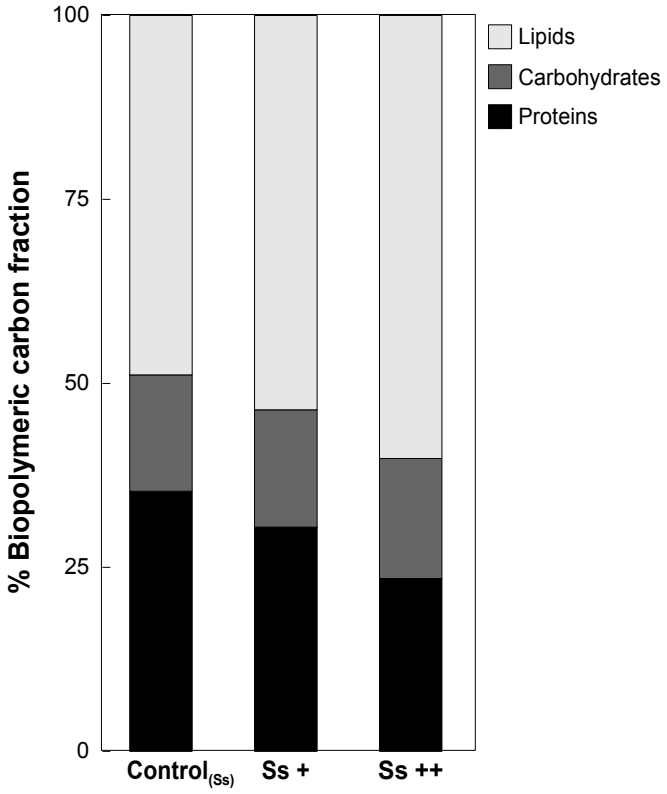

Fig. 4 Percentage of carbon content of carbohydrates, lipids and proteins in biopolymeric organic carbon at surficial sediment in Ss experiment.

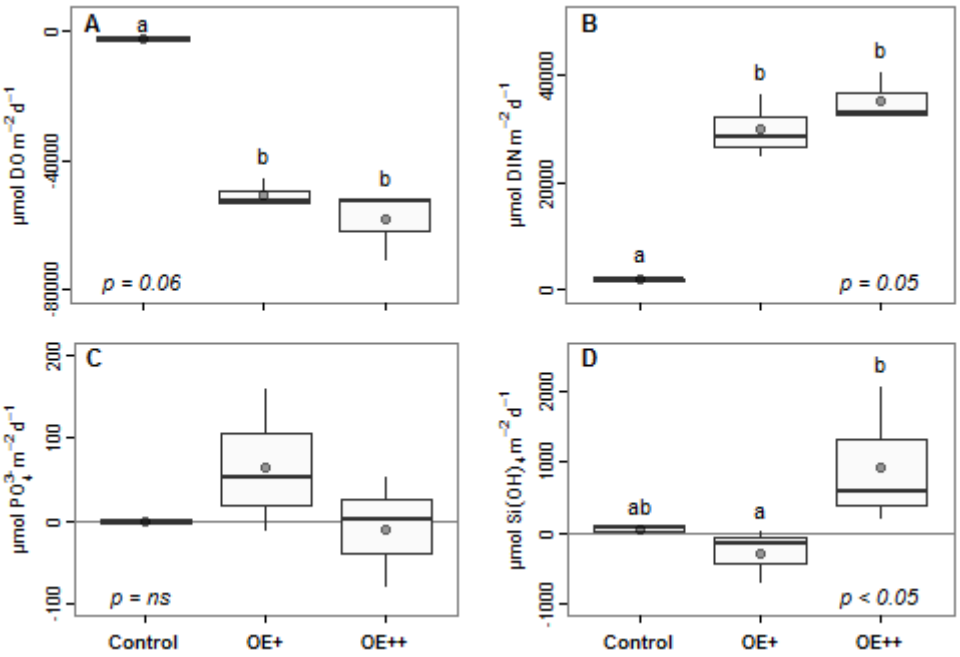

Fig. 5 Box plots of **A** DO, **B** DIN, **C** PO_4^{3-} and **D** $Si(OH)_4$ fluxes at sediment-water interface in the organic enrichment experiment. The box plots show the inter-quartile range with the median as a horizontal line. Error bars extend from the edge of the box to the highest and lowest values that are within 1.5 * IQR (interquartile range). Grey circles represent mean values. The letters over the box plots indicate the Kruskal Wallis test and multiple comparison of groups with Bonferroni adjustment. Medians with a different letter are significantly different at the significance level indicated on the plot.

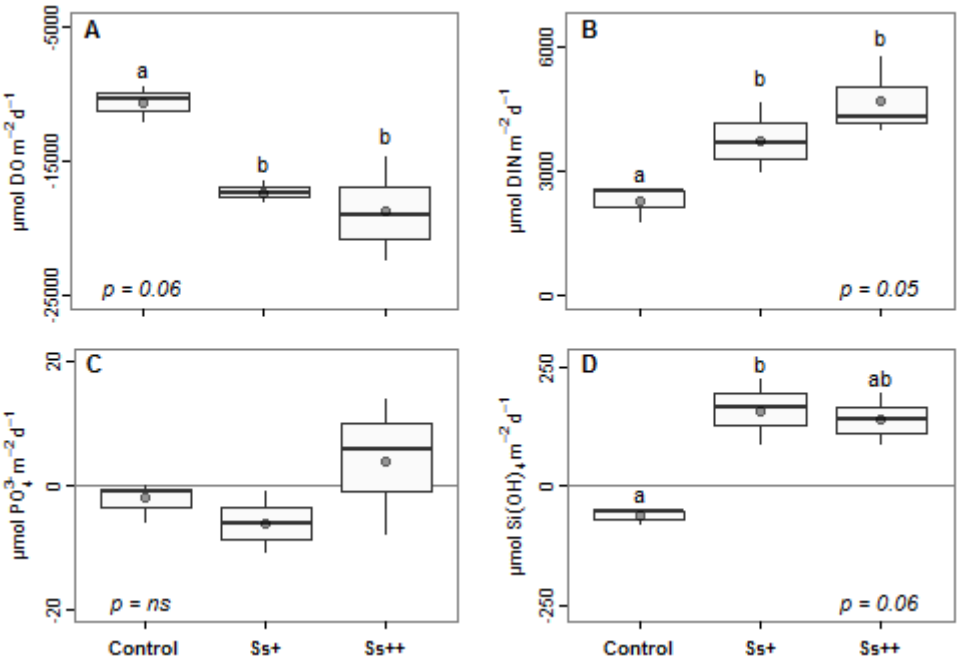

Fig. 6 Box plots of **A** DO, **B** DIN, **C** PO_4^{3-} and **D** $Si(OH)_4$ fluxes at sediment-water interface in the *Spisula subtruncata* experiment. The box plots show the inter-quartile range with the median as a horizontal line. Error bars extend from the edge of the box to the highest and lowest values that are within 1.5 * IQR (interquartile range). Grey circles represent mean values. The letters over the box plots indicate the Kruskal Wallis test and multiple comparison of groups with Bonferroni adjustment. Medians with a different letter are significantly different at the significance level indicated on the plot.

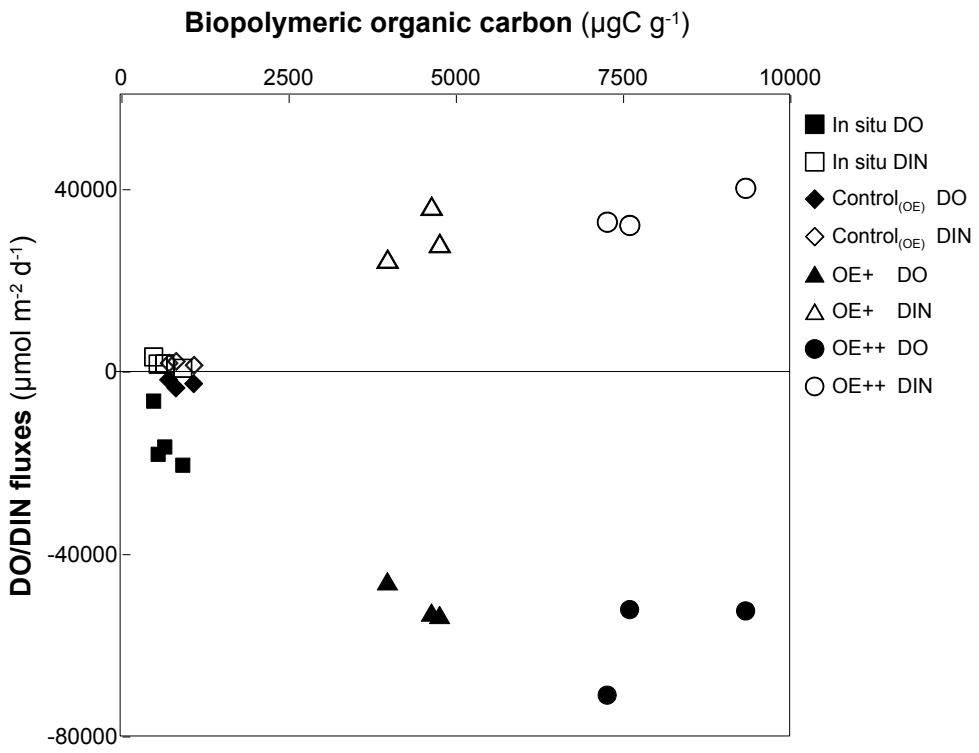

Fig. 7 Relationship between DO and DIN fluxes and biopolymeric organic carbon in OE experiment and *in-situ* incubations.