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Response of estuarine free-living nematode assemblages to organic enrichment: an experimental approach

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ABSTRACT: Organic enrichment, especially from anthropogenic sources, is one of the current threats to coastal marine biodiversity. Organic enrichment occurs mainly in sheltered soft bottoms, characterized by fine sediments, and results in multiple changes in the benthic habitat, including hypoxia and an increased concentration of compounds that are toxic to marine invertebrates. We report on the results of a microcosm-based experiment (duration = 30 d), quantifying the effects of organic enrichment on taxonomic and functional diversity of nematode assemblages from an open/closed coastal lagoon of South America (Rocha Lagoon, Uruguay). In open/closed lagoons, the input of organic matter becomes a major disturbance due the limitation in water renewal. In our experiment, enrichment led to reductions in abundance, richness and trophic diversity of the nematode assemblage. Rapid reductions in total abundance (after 4 d) were registered, while richness decreased only towards the end of the experiment (~30 d). Trophic changes were characterized by loss of predators/omnivores and dominance of selective deposit-feeders and epigrowth-feeders. By contrast, we did not find any selective effect of enrichment associated with life history traits (e.g. maturity index). Overall, these findings have 2 important implications for the conservation and monitoring of the health of coastal lagoons: first, monitoring of nematode assemblages at the genus level is sufficient to detect enrichment effects; second, an index of trophic diversity would be a good indicator of the effects of enrichment on natural communities.

KEY WORDS: Free-living nematodes · *Spirulina platensis* · Eutrophication · Laguna de Rocha · Uruguay

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INTRODUCTION

Nutrient enrichment of marine/estuarine areas can favour algal growth and lead to eutrophication, the occurrence of anoxia and hypoxia, fish kills (Glasgow & Burkholder 2000), loss or degradation of habitat for benthic organisms and a decrease in the number of fisheries. Eutrophication is considered one of the major stresses for aquatic environments, and it is characterized by excess biomass (Sampou & Oviatt 1991) and accumulation of refractory organic matter. Anthropic activities, including agricultural production and industrial and domestic effluents, modify the physicochemical and biological conditions of estuar-

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ine systems (Day et al. 1989, Perissinotto et al. 2010). These activities generally intensify the process of eutrophication, introducing inorganic nutrients that support the consequent increase in algal biomass and primary productivity in the water column (Cloern 2001, Pusceddu et al. 2009).

Organic enrichment is an important ecological process in marine/estuarine sediments (Kelly & Nixon 1984). Organic enrichment occurs more frequently in habitats characterized by fine sediments, low hydrodynamics and low dissolved oxygen concentration (Snelgrove & Butman 1994). Accumulation of organic compounds (labile and refractory) leads to changes in physical, chemical, biological and ecological features of sediments (Cloern 2001) and defines the quality and amount of food resources, and hence affects metabolic processes and mobility, as well as community structure, biodiversity and trophic structure (Grall & Chauvaud 2002). The labile fraction of the organic matter (carbohydrates, lipids and proteins) is easily digested and assimilated by heterotrophic organisms, and is the major energy source for benthic organisms (Ruhl et al. 2008). By contrast, the refractory fraction (e.g. humic and fulvic acids) are degraded more slowly and do not represent a favourable source of nutrition (Joseph et al. 2008).

At moderate levels of organic enrichment, benthic animals may show altered behavioural patterns, decreased feeding and reproductive activity, and changes in physiological functions (see reviews by Vernberg 1972, Herreid 1980). High levels of organic enrichment can produce important changes in communities and food webs, through e.g reductions of oxygen levels and modification of the chemical conditions of the sediment. Enrichment leads to reductions in diversity and community shifts, where the original community is replaced by one characterized by species resistant to organic pollution (Pearson & Rosenberg 1978, Hargrave et al. 2008, Venturini et al. 2012). At these levels, enrichment also leads to an impoverishment of the functional structure of the community (Pearson & Rosenberg 1978). Given that organic matter can cause changes at so many levels of biological organization, excessive input of organic matter can be considered a strong stressor (Pearson & Rosenberg 1978, Diaz & Rosenberg 2008).

In spite of the extensive coverage of the impact of organic enrichment on marine/estuarine ecosystems, the effect of the organic matter on the biota of coastal lagoons is not well documented or is underestimated (Kendall et al. 1995, Armenteros et al. 2010). Coastal lagoons are common coastal habitats, for instance in the Mediterranean Sea, the Gulf of Mexico and the Atlantic coast of North America, as well as the Atlantic coast of South America. Overall, lagoons comprise 13% of coastal regions globally (Bird 1994, Anthony et al. 2009). In coastal lagoons, the input of organic matter becomes a major disturbance because of the limitation in the capacity for water renewal (Urban et al. 2009). Coastal lagoons are considered particularly vulnerable to eutrophication, due to their restricted exchange with the adjacent sea, their shallow nature, and their high productivity. Lagoon eutrophication results from increasing human population densities along the lagoon coastline and from use of fertilizers for agriculture in their surrounding watershed (Cloern 2001).

Here, we quantified the effects of organic enrichment on taxonomic and functional diversity of assemblages of free-living nematodes from Rocha Lagoon (Atlantic coast of Uruguay, South America). The process of eutrophication existing in Rocha lagoon (see 'Study area' below for details) is representative of the situation being experienced by other coastal systems worldwide (Cloern 2001). We studied the effects of enrichment, through a laboratory experiment, using nematode assemblages as a model system. Laboratory experiments are considered an appropriate approach to study the effect of organic enrichment in marine and estuarine communities (Coull & Chandler 1992). Microcosm experiments enable the establishment of cause–effect relationships (Nilsson et al. 1991) and can be used to determine which organisms are indicators of disturbances (Heip et al. 1985, Coull 1988).

Free-living nematodes are excellent organisms for laboratory experiments purposes, due to their small size, short life cycle, quick response to environmental changes and resistance to sediment manipulation (Warwick et al. 1988). Although the manipulation of field sediment leads to a disruption of the interstitial environment, the response of nematodes has been successfully separated from the 'microcosm effect' in a procedural control in studies of effects of xenobiotics (Austen & McEvoy 1997, Hedfi et al. 2007), sedimentation (Schratzberger et al. 2000) and organic enrichment (Schratzberger & Warwick 1998, Armenteros et al. 2010). Several ecological factors such as habitat type (e.g. sandy beaches, estuaries, etc.), the origin of organic inputs and the intensity of human disturbances affect spatial distributional patterns of free-living nematodes (Schratzberger et al. 2008).

We studied the effect of enrichment on taxonomic diversity at the species/genus levels as well as functional diversity, quantified in terms of feeding types and life strategies. We expected that by combining

taxonomic and functional diversity we would obtain a better understanding of the structural components and the functioning of the benthic community (Norling et al. 2007). In particular, for nematodes, the relationships between the functional attributes (e.g. trophic responses) and organic matter amount and quality are not well understood yet. Therefore, our experimental approach also offers the possibility to establish the taxonomic and functional responses of nematodes to enrichment. The patterns observed in experimental approaches may contribute to a better understanding and prediction of the patterns observed in nature. In particular, we hypothesized that organic enrichment would lead to reductions in taxonomic diversity and an increase in the abundance of nematodes that are tolerant to disturbance. We also expected low trophic diversity, as well as the dominance of organisms with short life cycles.

MATERIALS AND METHODS

Environmental set-up

Experimental surface sediments and experimental nematodes were collected during January 2015 in the south of Laguna de Rocha, Uruguay (34° 39′ 47.42″ S, 54° 13′ 47.36″ W, see Fig. 1 in Kandratavicius et al. 2015). Rocha Lagoon is a choked type lagoon (Kjerfve & Magill 1989, Conde et al. 2000) with an area of 7304 ha, shallow and with an intermittently open-closed connection with the Atlantic Ocean. The connection with the ocean takes place several times per year, when depth increases and when the sandbar is breached by wave action (Conde & Rodríguez-Gallego 2002).

Among the major ecological problems of Rocha Lagoon is the recent eutrophication, probably caused by land use and the input of domestic effluents (Rodríguez-Gallego et al. 2008). Industrial activity is limited and is mainly stockbreeding, but the lagoon receives anthropogenic inputs from the city of Rocha and the municipal slaughterhouse (via Rocha Stream) and has received further inputs in the past from a fish-processing plant and agriculture (Arocena et al. 2000). Using sedimentary organic matter and biochemical composition, Pita et al. (2017) recently classified Rocha Lagoon as eutrophic.

Kandratavicius et al. (2015) found that meiofauna is dominated by nematodes (63%), copepods (15%) and ostracods (7%). Nematodes were significantly more abundant in summer and in fine sand, which was more common in the inner zones of Rocha lagoon.

Sampling and microcosm set-up

Sediment samples and fauna were collected by hand because of the shallow habitats (<1 m) in a location known as 'old bar' (34° 39′ 47.20″ S, 54° 13′ 50.41″ W) characterized by fine sediments (69% mud, fine and medium sand), low organic content (~1.32%), salinity 18.9 (average in summer) and high temperatures (28°C) (Giménez et al. 2014, Kandratavicius et al. 2015). Five plastic cores (2.7 cm internal diameter) were buried to 10 cm depth in the sediment in order to take samples for the description of community structure. In addition, 3 surface sediment samples (1 cm depth, approximately 300 g) were collected for the estimation of total organic matter, chlorophyll *a* and organic biopolymers (total lipids, carbohydrates and proteins). All of these samples were considered as field controls.

A key point for the validity of the experimental study is that it should be as homogeneous as possible across experimental units, and the effects of treatments must be stronger than the 'microcosm effect', i.e. the effect of manipulation of sediments (Austen & McEvoy 1997). We carefully collected approximately 15 l of surface sediment to set-up the experimental units or microcosms. The fresh sediment collected in the lagoon was transported to the laboratory, stored in 2 containers with aeration for approximately 24 h. Thereafter, the sediment was gently homogenized with a spoon, and 5 random aliquots of sediment were checked for the presence of living nematodes, identified as individuals moving in the sediment. Each microcosm was considered to be an independent experimental unit and consisted of a 250 ml glass beaker with 150 ml of sediment (resulting in a 4 cm layer of sediment) and lagoon water with an individual aerator. The microcosms were placed on a lab table and kept under natural climatic conditions, with temperatures ranging from 20–25°C and a summer light:dark cycle of temperate regions (about 14:10 h light:dark). In total, 77 microcosms were made: 50 microcosms were used to evaluate the response of the nematode communities to organic enrichment, and 27 microcosms were used to evaluate changes in chlorophyll *a* (chl *a*), total organic matter and biopolymers (Fig. 1).

The increased organic matter treatment was created by adding commercial microalgae *Spirulina platensis* particulate. The biopolymeric composition of *S. platensis* was 60% protein, 30% carbohydrates and 10% lipids, which was similar to the proportions reported from natural populations (Ríos et al. 1998). The chl *a* content was 87 µg g^{-1} dry weight. The chl *a*

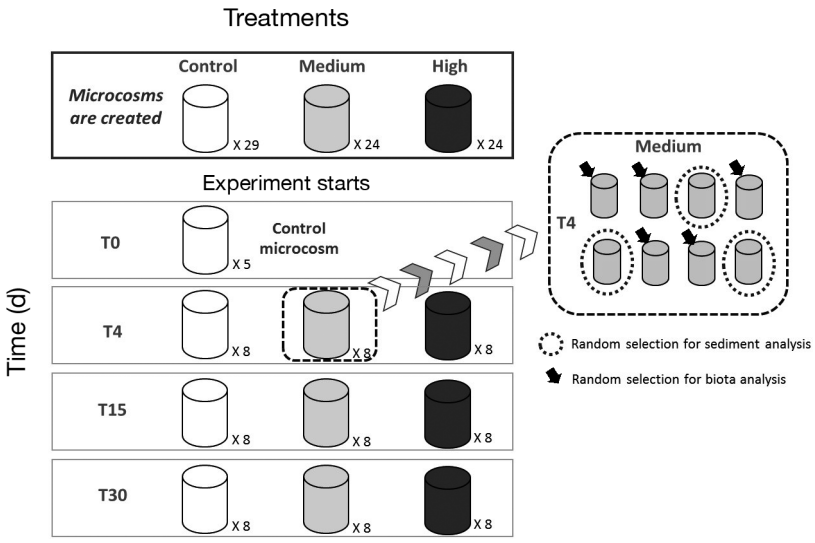

Fig. 1. Experimental design. Three different treatments were applied: Medium (2.5 g *Spirulina platensis*), High (5 g *S. platensis*) and Control (no *S. platensis*). On Day 0, 5 microcosms were removed from the control treatment to analyze the initial structure of the nematode assemblages (microcosm control). On Days 4, 15 and 30, we randomly extracted 24 microcosms (8 replicates treatment^{-1}). From 8 replicated microcosms treatment^{-1}, 5 were taken for the analysis of nematode structure and 2 for the chemical analysis of sediment

content in field was 10 µg l^{-1} (Conde et al. 2003); considering those results, we modified the method of Armenteros et al. (2010) in order to produce 3 treatments as follows: (1) High level: addition to the microcosm of 5 g of *S. platensis* equivalent to 43.5 µg l^{-1} chl *a*, around 4 times the field concentration (24 microcosms = 15 for nematode community analysis + 9 for sediment analysis); (2) Medium level: addition of 2.5 g of *S. platensis* equivalent to 21.75 µg l^{-1} chl *a*, around twice the field concentration (24 microcosms = 15 for nematode community analysis + 9 for sediment analysis) and (3) Control: no addition (29 microcosms = 20 for nematode community analysis + 9 for sediment analysis).

At the beginning of the experiment (Time 0, T_0), 5 microcosms of the control treatment were used to analyse the structure of the nematode community (microcosms are destroyed during the sampling and thus are used only once). At 4, 15 and 30 d, 5 microcosms from each treatment were used to analyse the structure of the nematode community, and 3 microcosms were used to analyse the organic matter, biopolymers and chl *a* content (Fig. 1). The dissolved oxygen concentration and temperature were measured daily in the water matrix (measurements done with an O_2 microsensor Unisens® OX50 and YSI® multi-parameter, respectively).

Sample processing

The content of each microcosm was used to analyze different attributes of sediment. Photosynthetic pigments (chl *a* and phaeopigments) were analyzed according to Lorenzen (1967), modified by Sündback (1983) for sediments. Total organic matter (OM) was analyzed based on Byers et al. (1978) and expressed as a percentage (%). The biochemical composition of OM was analyzed following the protocols described by Danovaro (2010). Total protein (PRT) analysis was conducted according to Hartree (1972), modified by Rice (1982) to compensate for phenol interference. Total carbohydrates (CHO) were analyzed according to Gerchakov & Hatcher (1972). Total lipids (LIP) were extracted by ultrasonication with a mixture of chloroform:methanol (1:2 v/v) and analyzed following the protocol described by Marsh & Weinstein (1966). Blanks for each analysis were performed with precombusted sediment (450°C, 4 h). PRT, CHO and LIP concentrations were expressed as bovine serum albumin, glucose and tripalmitine equivalents, respectively. PRT, CHO and LIP concentrations were converted to carbon equivalents assuming a conversion factor of 0.49, 0.40 and 0.75 µg, respectively (Fabiano & Danovaro 1994). The sum of PRT, CHO and LIP carbon equivalents is reported as the biopolymeric carbon (BPC) and used as a reliable estimate of the labile fraction of OM (Fabiano et al. 1995) and to classify the trophic status of the sediments. Also, the PRT:CHO ratio and the CHO:LIP ratio were calculated and used as indicators of the status of biochemical degradation processes (Galois et al. 2000).

In order to sample nematodes, the content of each microcosm was washed between a 500 and a 63 µm sieve using filtered water. To extract the meiofauna from the sediment fraction, retained on the 63 µm sieve, we applied a flotation technique using Ludox HS 40 colloidal silica (1.18 g cm^{-3}) and centrifugation (Heip et al. 1985, Vincx 1996). This process was repeated 3 times, whereby each time the supernatant Ludox containing the meiofaunal organisms was decanted and washed. The final washed and ex-

tracted sample was then preserved in 4% formaldehyde, and a small amount of Rose Bengal was added to facilitate identification. Using a binocular loupe, 100 nematodes were randomly picked out of each microcosm and mounted on glass slides for genus identification under the microscope (Somerfield & Warwick 1996) using pictorial keys (Platt & Warwick 1983, 1988, Warwick et al. 1998).

Before assembly onto glass slides, nematodes were placed in a solution of glycerol–ethanol and allowed to evaporate in a desiccator so that the nematodes remained in glycerin, facilitating the observation of their structures.

Structure of nematode assemblages and biological/functional traits

Richness (as number of genera) and abundance of nematodes per genus was determined for each microcosm. Each one was classified according to its life strategy on the spectrum of coloniser–persister (c-p score: Bongers 1990, Bongers et al. 1991). The scale range is defined from extreme colonisers (c-p score = 1) to extreme persisters (c-p score = 5). The maturity index (MI) of the community was calculated using the formula (Bongers et al. 1991):

$$MI = \Sigma (v_{(i)} \times f_{(i)}) \quad (1)$$

where $v_{(i)}$ = the c-p value of genus i and $f_{(i)}$ = the relative frequency of genus i.

Additionally, nematode genera were assigned to feeding types according to Wieser's (1953) classification based on the morphology of the buccal cavity: selective deposit-feeder (1A), nonselective deposit-feeder (1B), epigrowth feeder (2A) and omnivore/predator (2B). This classification was used to calculate the index of trophic diversity (ITD, Heip et al. 1985), calculated as:

$$ITD = \Sigma \theta^2 \quad (2)$$

where θ is the percentage contribution of each trophic group according to Wieser (1953). ITD values range between 0.25 (high trophic diversity: the 4 groups have a representation of 25%) and 1.0 (low trophic diversity: a single trophic group dominates at 100%).

Data analysis

Multi- and univariate techniques were used for data analysis using the software PRIMER 6.0.2 (Clarke & Gorley 2006) and STATISTICA 10.0 from StatSoft. If needed, data were transformed and re-checked to determine if parametric assumptions were applicable. Comparisons to test changes in biota and trophic status of sediment (based on OM, chl *a*, phaeopigments and biopolymers) between control groups were done in order to assess the 'microcosm effect' (abiotic and biotic changes due to experiment artifacts) using a 1-way ANOVA with 5 levels: field control, T_0 (microcosm control) and controls at Time 4, 15 and 30 d. If the differences in ANOVA were significant, 2 comparisons using least square means (*t*-test) were performed: (1) field vs. T_0 to test the 'microcosm effect', i.e. the differences between field and the experimental conditions; and (2) T_0 vs. controls at 4, 15 and 30 d (C_4, C_{15}, C_{30}) to test the temporal changes in the microcosm controls. The results of these tests allow establishing the validity of the experimental setting.

Treatment effects (i.e. among levels of enrichment) were evaluated first for the trophic status of sediment and separately for a possible effect on the trophic response of nematodes (MI and ITD) or structural changes in the assemblage (richness and abundance per genus). Those tests were carried out through a 2-way factorial ANOVAs where the treatments of OM input (control, medium and high) and time (4, 15, 30 d) were used as factors. Treatment effects of dissolved oxygen were evaluated with a repeated measures ANOVA, where time was the repeat factor.

Responses of multivariate structure of nematode assemblages to treatments were tested with permutation-based ANOVA, using PERMANOVA (Anderson et al. 2008). Data were square root transformed in order to downweigh the contribution of dominant species. Similarity matrices were built using the Bray-Curtis index and permutations were run on the reduced model; reported p-values are based on Monte Carlo tests, and results were considered significant at $p < 0.05$. The SIMPER procedure was applied to identify genera which contributed the most to similarity/dissimilarity across treatments and/or times.

RESULTS

Abiotic component

Validation of the experimental setting

On average, chl *a*, phaeopigments and CHO (Figs. 2 & 3) were significantly higher in the control T_0 than in the field (1-way ANOVA and *t*-test: chl *a*: $F_{4,10} = 23.73$, $p < 0.001$, phaeopigments: $F_{4,10} = 53.95$

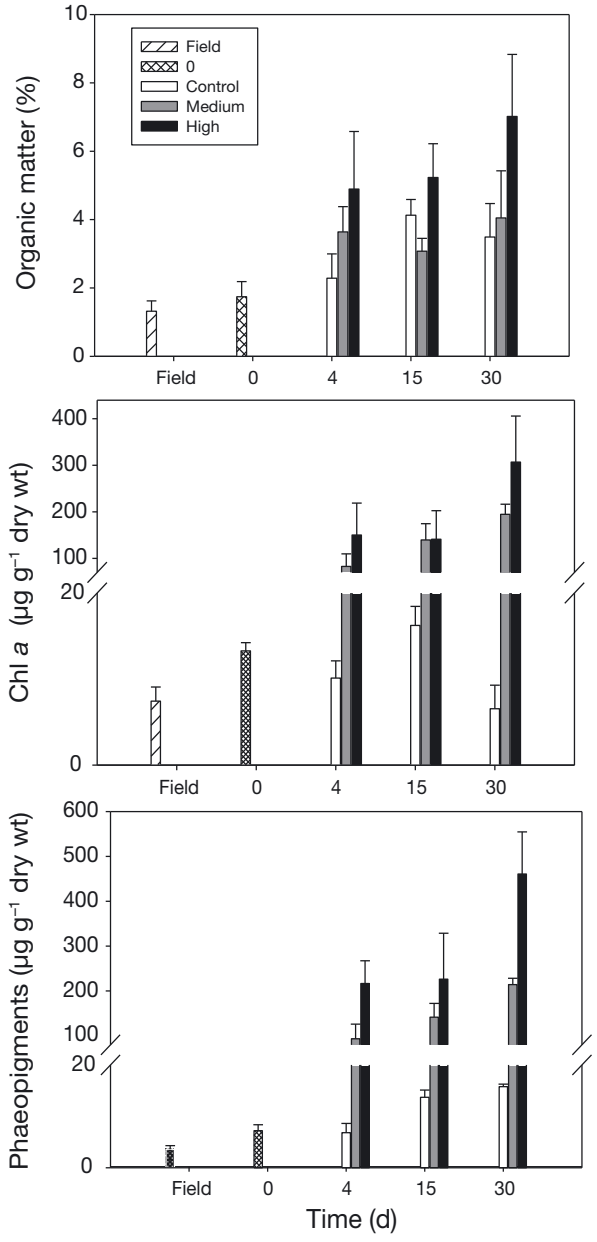

Fig. 2. Means and SD of abiotic factors measured from a field site, at Time 0 and in microcosm treatments (control, medium and high levels of enrichment) at different times (4, 15 and 30 d after the onset of the experiment)

$p < 0.001$, carbohydrates: $F_{4,10} = 5.26$ $p < 0.001$, Table 1). These variables had an important increase in T_0 with respect to field samples (Table 2). The percent increase was 78% in chl *a*, 100% in phaeopigments and 25% in CHO.

Only the phaeopigments, chl *a* and OM increased significantly over time in the controls (Table 1), where values were more than twice those found in the field. The PRT:CHO ratio was <1 in field samples, T_0 and controls, while in the treatments, recorded values were >1. The CHO:LIP ratio was >>1 in field samples, T_0, controls and both treatments.

Enrichment

There was a significant and important enrichment in terms of OM, chl *a* and phaeopigments (Fig. 2, Tables 2 & 3). The addition of *Spirulina platensis* at both densities resulted in significant increases in chl *a* and phaeopigments, while the maximum levels of OM were clear only under high levels of *S. platensis*. The significant increase in phaeopigments occurred progressively from Day 4 to Day 30 (post hoc test: $T_4 < T_{15} < T_{30}$; see Table S1 in the Supplement at www.int-res.com/articles/suppl/m602p117_supp.pdf).

The addition of *S. platensis* also increased the levels of BPC, LIP and PRT (Fig. 3, Table 2 & 3) while CHO levels were not significantly affected. PRT levels increased progressively (significant interaction of time × treatment, Table S1), while those of BPC and lipids were established quickly, especially after addition of *S. platensis* in the high-density treatment.

The addition of *S. platensis* resulted in a significant decrease in dissolved oxygen (Table 3, Fig. 4). Its levels reached the limit of hypoxia (2 mg l^{-1}) after ~1 d and tended to recover after around 10 d; recovery took place more slowly after addition of *S. platensis* in the highest treatment (Fig. 4, see Table S1). In addition, enrichment led to hypoxia.

Overall, our results (increased levels of pigments, OM, LIP, PRT and BPC) validated the experimental setting. Hence, in the remainder of this article, we call the treatments 'control', 'medium level of enrichment' (= addition of 2.5 g of *S. platensis*) and 'high level of enrichment' (= addition 5 g of *S. platensis*).

Responses of the nematode assemblage

A comparison of univariate measures of assemblages between field and control samples on Day 0 indicates how much the assemblages in the experimental conditions mimic those of the natural environment (Fig. 5). In general, nematode assemblages were similar in field, control microcosms and T_0. There were no significant differences in the log-transformed number of nematodes ($F_{4,20}$ =1.641, $p > 0.05$). Also, nematode abundance did not vary significantly at the end of the experiment in comparison with T_0.

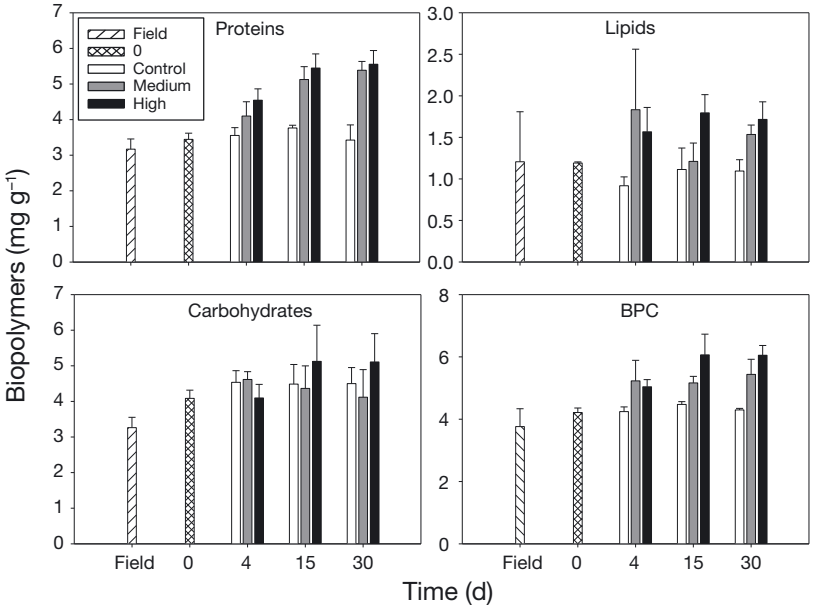

Fig. 3. Means and SD of biopolymers (BPC: biopolymeric carbon) measured from a field site, at Time 0 and in microcosm treatments (control, medium and high levels of enrichment) at different times (4, 15 and 30 d after the onset of the experiment)

Nineteen genera of free-living marine/estuarine nematodes were recorded in our study (Table S2 in the Supplement). In general, the assemblages of genera were similar in field and control microcosms at T_0. There were no significant differences in the richness of genera (Table 4, Fig. 6) or in the number of individuals per genus, except in the case of rare genera (*Neochromadora*, *Oncholaimus*, *Oncholaimellus*, *Halalaimus*, *Kosswigonema*, *Antomicron*, *Daptonema*, *Leptolaimus*, morphotype 1, *Theristus*) with higher abundance in the field with respect to T_0, and *Sabatieria* with lower abundance in the field in compared to T_0 (Table 4, Fig. 7). The dominant genus (*Pseudochromadora*) was the same in the field control and T_0.

The number of genera (richness) was significantly different among treatments, with lower values in medium and high treatments compared to control. There were no significant changes over time in genus richness in the controls, but in the treatments, both the medium and high treatment suffered a decrease in the number of genera over time (Table 4).

There was a significant multivariate effect of enrichment and time on the nematode assemblage (PERMANOVA significant Time × Treatment interaction: pseudo-F = 2.735, Monte Carlo p = 0.0006; 9920 permutations), showing that differences among treatments depended on the sampling time (Tables 5 & S1). After 15 d, significant differences were found only between medium and high enrichment treatments (t = 1.599, Monte Carlo p = 0.05, 126 permutations); however, after 30 d, differences between control versus both medium and high enrichment treatments were significant (Monte Carlo p = 0.0006, p = 0.006, 126 permutations) (Tables 5 & S1).

The SIMPER procedure indicated that the same genera contributed to similarity within groups of controls at 4, 15 and 30 d: *Pseudochromadora* and *Terschellingia*. In addition, the average dissimilarity between controls at different times did not exceed 20%. The pairwise comparisons between T_0 and the field sample also showed a significant difference, but the R value was low (R = 0.28, p = 0.04, 126 permutations), suggesting a small effect (Fig. 8, average dissimilarity: 38%). The most marked differences (average dissimilarity 50%) were observed in the pairwise comparisons between enriched treatments at 4 and 30 d.

There were significant effects of enrichment on the number of individuals in 6 out of 10 genera (exceptions were *Oxystomina*, *Sabatieria*, *Paradontophora*

Table 1. Results of least square means (t-test) of field samples vs. Time 0 (T_0) to test the differences between field and experimental conditions; and of T_0 vs. controls at Time 4, 15 and 30 d (C_4, C_{15}, C_{30}) to test the temporal changes in the microcosm controls. This test was applied to variables with significant 1-way ANOVA. In all cases, df = 4. OM: organic matter, CHO: carbohydrates. *p < 0.05

	Field vs. T_0		T_0 vs. C_4		T_0 vs. C_{15}		T_0 vs. C_{30}	
	t	p	t	p	t	p	t	p
OM	1.37	0.242	−1.11	0.327	6.45	0.003*	−2.81	0.04*
Chl *a*	3.35	0.028*	−4.22	0.01*	8.32	0.001*	−1.04	0.35
Phaeopigments	4.63	0.009*	0.31	0.77	6.17	0.003*	−11.7	<0.001*
CHO	4.17	0.014*	−1.52	0.20	2.91	0.04	−1.07	0.34

Table 2. Size effects expressed as % increase (Time 0 [T_0] vs. field samples and T_0 vs. enrichment treatments, where M: medium enrichment, H: high enrichment; 4, 15 and 30 represent time in days) in chl *a*, phaeopigments, organic matter (OM) and biopolymers (CHO: carbohydrates, PRT: proteins, LIP: lipids, BPC: biopolymeric carbon)

	Chl *a*	Phaeopig	OM	CHO	PRT	LIP	BPC
T_0 vs. Field	78	100	32	25	9	−1	12
T_0 vs. M4	524	1197	109	13	19	52	39
T_0 vs. M15	953	1867	76	7	49	0.4	37
T_0 vs. M30	1367	2877	132	1	56	27	45
T_0 vs. H4	1032	2911	181	0.3	32	30	34
T_0 vs. H15	964	3044	200	25	58	49	61
T_0 vs. H30	2214	6303	303	25	61	42	61

and *Terschellingia*: Table 3). In the genera *Anonchus* and *Anoplostoma*, a decrease in the number of individuals was observed in both enriched treatments (high and medium) compared to the control (Fig. 8). For *Viscosia* and *Paralinhomoeus*, the number of individuals decreased in the medium enrichment treatment with respect to the control but increased under high level of enrichment compared to medium (Fig. 8). All genera significantly decreased in abundance over the experimental time, especially under high enrichment; by contrast, *Pseudochromadora* increased in abundance with time, especially under high enrichment conditions (Table 4, Fig. 8).

Community maturity and trophic diversity

The MI and ITD did not show significant differences among field samples and controls on Day 0 (Table 4, Fig. 9). There was, however, a significant temporal variation and a significant effect of organic enrichment on trophic structure of assemblages depending on time (significant interaction of time × enrichment: Table 4), shown from ITD values driven by changes in deposit-feeders (groups 1A and 1B) as well as epigrowth feeders (2A). At the end of the experiment (T_{30}), the trophic diversity decreased, which was driven by a decrease in the percentage associated with deposit-feeders (1B) and predators/omnivores (2B) accompanied by an increase in the proportion of epigrowth feeders (Fig. 9). In addition, a general decrease in trophic diversity and the increase of MI values over time (significant time effect: Table 4, Fig. 9) were observed in both treatments. However, the increase in MI (to values of 3 or 4), indicates a lack of disturbance effects.

DISCUSSION

In assessing the effect of enrichment on nematode assemblages, we used a microcosm approach. We therefore increased our capacity to establish cause–effect relationships at the cost of losing realism. We minimized effects related to the construction of laboratory assemblages, the so-called microcosm effect (leading to microhabitat homogenization, temporal hypoxia and mortality of sensitive species): at T_0, assemblages did not differ from those in the field (Fig. 9), and both field conditions and the control at the beginning of the experiment had similarly aged

Table 3. Results of statistical comparisons of univariate measures of abiotic components. For all variables except dissolved oxygen, data were analyzed with a standard 2-way crossed ANOVA, considering treatment and sampling time as factors. Oxygen data were analyzed using within-subject (repeated measures) ANOVA considering time as a within-subject factor and treatment as a between-subject factor; in this case, there are 3 separate analyses by time comparing daily measures of oxygen level. OM: organic matter, BPC: biopolymeric carbon. *$p < 0.05$

F	Treatment			Time			Treatment × Time		
	df	*F*	p	df	*F*	p	df	*F*	p
OM	2,18	12.37	<0.001*	2,18	2.78	0.089	4,18	1.48	0.25
Chl *a*	2,18	85.03	<0.001*	2,18	7.47	0.04*	4,18	4.1	<0.016*
Phaeopigments	2,18	344.7	<0.001*	2,18	21.3	<0.001*	4,18	1.7	0.202
BPC	2,18	30.01	<0.001*	2,18	3.4	0.056	4,18	2.12	0.121
Proteins	2,18	58.69	<0.001*	2,18	13.96	0.00*	4,18	3.86	0.02*
Lipids	2,18	10.68	<0.001*	2,18	0.16	0.855	4,18	1.82	0.17
Carbohydrates	2,18	1.007	0.385	2,18	0.354	0.707	4,18	1.41	0.271
O_2 Time 4	2,12	529.34	<0.001*	4,48	24.545	<0.001*	8,48	19.607	<0.001*
O_2 Time 15	2,12	171.82	<0.001*	14,168	11.779	<0.001*	28,168	8.921	<0.001*
O_2 Time 30	2,12	53.542	<0.001*	27,324	46.642	<0.001*	54,324	12.788	<0.001*

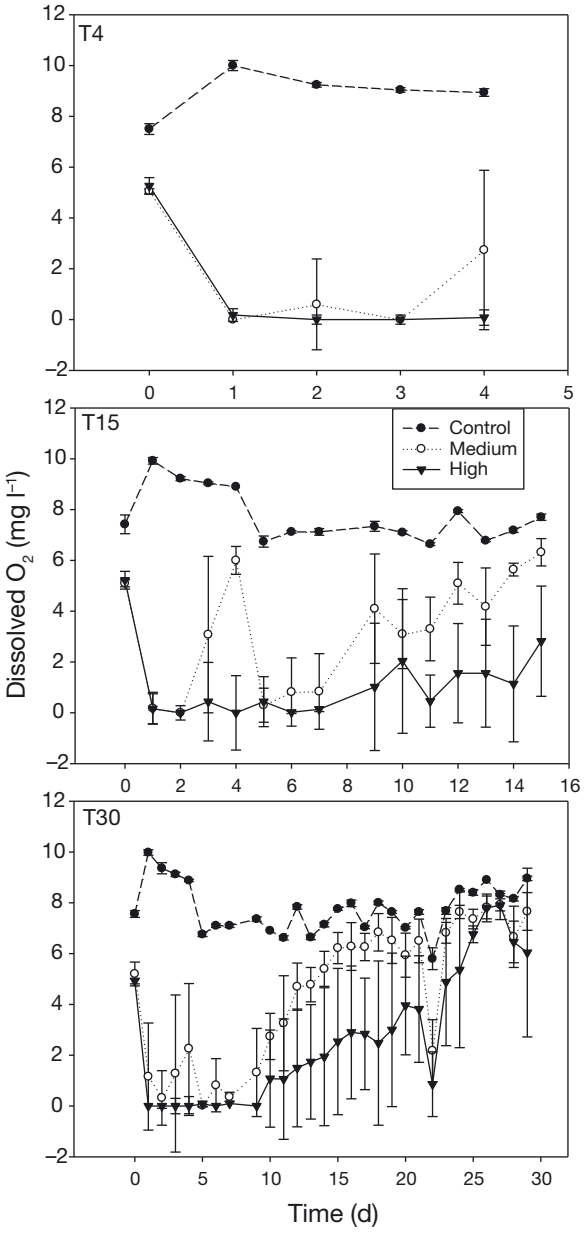

Fig. 4. Means and SD of dissolved oxygen from microcosm treatments (control, medium and high levels of enrichment) sampled 4, 15 and 30 d after the onset of the experiment (T4, T15 and T30, respectively)

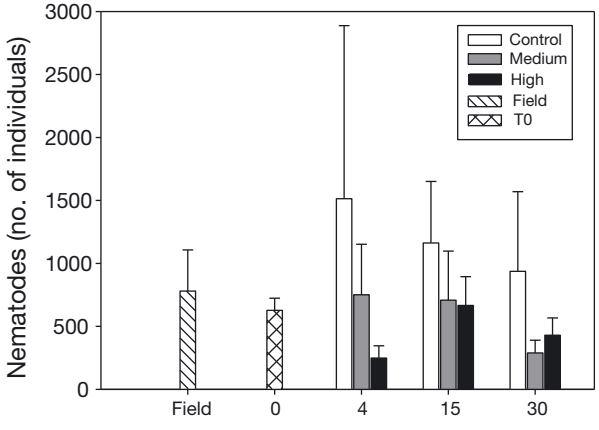

Fig. 5. Means and SD of nematode abundance measured from a field site, at Time 0 and in microcosm treatments (control, medium and high levels of enrichment) at different times (4, 15 and 30 d after the onset of the experiment)

and degraded sedimentary OM (PRT:CHO ratio <1: Dell'Anno et al. 2002) and low-quality OM (CHO:LIP ratio ≫1). The fact that the enrichment effects were stronger than the effects of sediment disruption (see Table 1) validated our approach according to the criteria recommended by Austen & McEvoy (1997). The environmental variables and the nematode assemblages varied little in the controls. The little variation in the nematode assemblage between field samples, T_0 and controls was consistent with the fact that estuarine nematodes are robust to laboratory manipulation (Austen & McEvoy 1997) and elicit a minimal 'microcosm effect' (e.g. Schratzberger et al. 2000, Hedfi et al. 2007). Overall, microcosm effects, if they existed, would have led to immediate changes in the environment and biota. We conclude that the observed reductions in abundance, richness of genera and trophic richness and changes in trophic structure (loss of predators/omnivores and a dominance of selective deposit-feeders and epigrowth-feeders) in responses to enrichment as well as with reductions in oxygen concentration, are likely to occur under natural conditions given that we started our experiment with realistic nematode assemblages. However, we recognize that further confirmation is needed through monitoring of natural assemblages and field experiments.

The experimental treatments were accurate in recreating organic enrichment and its consequences, in terms of trophic status of sediment and hypoxia. In our experiment, we simulated an important input of labile OM with the addition of *Spirulina platensis*. Hence, as particles sank in the experimental units, there was an increase in chl *a* and PRT (detected as a significant interaction between enrichment by time; Table 2). There was also an increment in phaeopigments 4 d after the beginning of the experiment, as well as a change in the age of the OM, from aged to live/fresh, as quantified from the ratio of PRT:CHO (<1 in controls and >1 in enriched treatments; Danovaro et al. 1993). Equally, the quality of OM increased as indicated from the increase in levels of BPC (Fabiano et al. 1995) and low CHO:LIP ratios

Table 4. Results of statistical comparisons of univariate measures of nematode assemblages, based on the 2 types of ANOVA used: 1-way (field collected samples vs. Time 0 [T_0]) and 2-way crossed. ITD: index of trophic diversity. Feeding types are 1A: selective deposit-feeder, 1B: non-selective deposit-feeder, 2A: epigrowth feeder and 2B: omnivore/predator. *p < 0.05

Factor	Treatment		Time		Treatment × Time		Field vs. T_0	
	$F_{2,36}$	p	$F_{2,36}$	p	$F_{4,36}$	p	$F_{1,8}$	p
Nematodes	17.11	<0.001*	4.8	0.014*	3.95	0.009*	1.25	0.297
Anonchus	13.38	<0.001*	10.44	<0.001*	1.08	0.381	2.89	0.128
Anoplostoma	5.01	0.012*	15.4	<0.001*	4.32	0.006*	5	0.056
Oxystomina	2.985	0.063	0.002	0.998	1.791	0.152	2.51	0.152
Pseudochromadora	12.04	<0.001*	27.27	<0.001*	5.86	0.001*	0.256	0.626
Paradontophora	2.537	0.093	6.805	0.003*	2.154	0.094	4.5	0.067
Paralinhomoeus	4.544	0.017*	1.931	0.16	0.504	0.733	1.76	0.221
Sabatieria	1.95	0.156	11.51	<0.001*	5.6	0.001*	9.09	0.017*
Terschellingia	0.964	0.391	1.581	0.222	2.568	0.054	0.61	0.459
Viscosia	7.14	0.002*	26.87	<0.001*	1.03	0.404	0.276	0.614
Rare genera	3915	0.029*	5.06	0.012*	1.70	0.17	7.86	0.023*
Richness of genera	9.25	<0.001*	17.79	<0.001*	3.37	0.019*	3.368	0.104
ITD	12.56	<0.001*	32.64	<0.001*	7.52	<0.001*	0.255	0.627
1A	2.269	0.118	2.295	0.115	4.02	0.009*	0.116	0.745
1B	3.39	0.045*	15.53	<0.001*	3.84	0.011*	0.857	0.39
2A	6.68	0.003*	18.78	<0.001*	4.48	0.005*	0.437	0.533
2B	7.04	0.003*	27.88	<0.001*	1.21	0.325	0.023	0.884
MI	0.353	0.705	7.412	0.002*	1.941	0.125	0.191	0.63

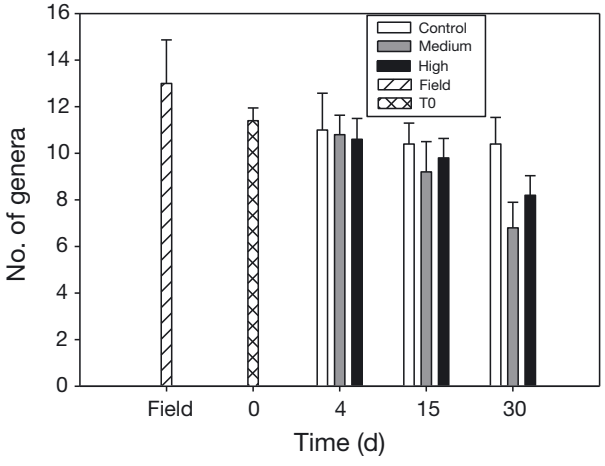

Fig. 6. Richness of genera (mean and SD) of nematode assemblages in sediments from a field site, at Time 0, and in microcosm treatments (control, medium and high levels of enrichment) at different times (4, 15 and 30 d after the onset of the experiment)

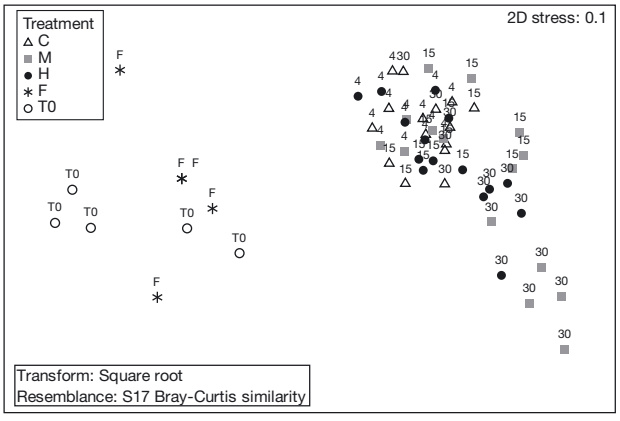

Fig. 7. Non-metric multidimensional scaling ordination of samples based on square-root transformed data of the density of nematode genera in sediment from: a field site (F), at Time 0 (T0), and in microcosm treatments (C: control, M: medium and H: high levels of enrichment). Numbers above symbols indicate days after the onset of the experiment

Table 5. PERMANOVA testing for responses of nematodes to enrichment (control, medium and high) and time. Perm: number of permutations. *p < 0.05

	df	SS	MS	Pseudo-*F*	p (Monte Carlo)	Perm.	p(perm)
Treatment	2	1799	899.5	5.5485	0.0001*	9936	0.0001*
Time	2	3394.5	1697.2	10.469	0.0001*	9930	0.0001*
Treatment × Time	4	1773.8	443.45	2.7354	0.0006*	9920	0.0003*
Residual	36	5836.2	162.12				
Total	44	12803					

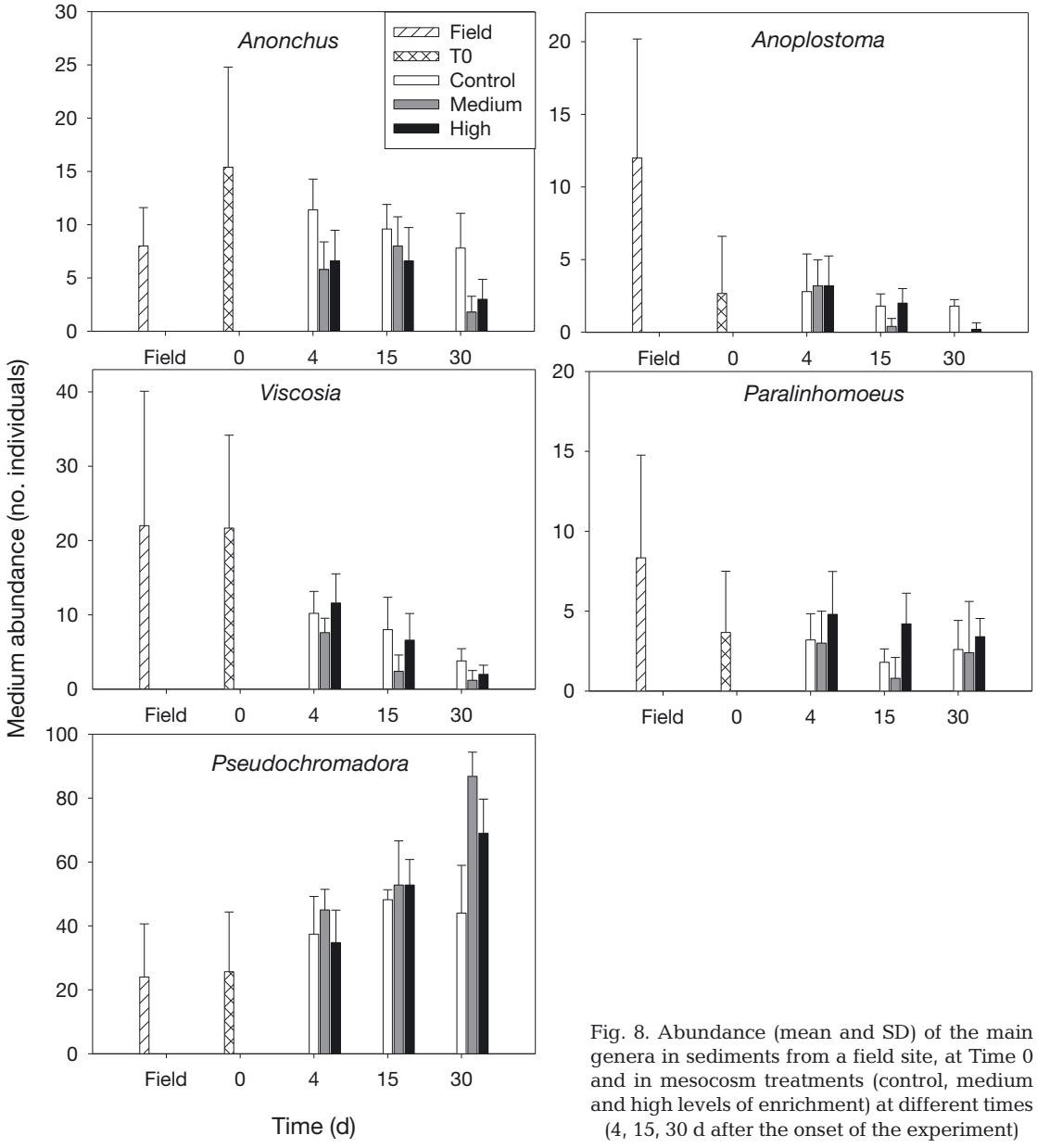

Fig. 8. Abundance (mean and SD) of the main genera in sediments from a field site, at Time 0 and in mesocosm treatments (control, medium and high levels of enrichment) at different times (4, 15, 30 d after the onset of the experiment)

(Joseph et al. 2008) driven by an increase in the concentration of LIP. CHO:LIP ratios (≫1) indicated that that the input of fresh OM had a low nutritional level, albeit higher than that registered in controls. Enrichment also resulted in sediment hypoxia (oxygen concentrations < 2.8 mg l^{-1}: Diaz & Rosenberg 1995), consistent with previous studies (Armenteros et al. 2010). The magnitude of hypoxia was higher over the first 15 d of the experiment (detected as a significant interaction of enrichment with time: Table 2). By contrast, most environmental variables varied little over time in the controls, with the exception of increases in OM and phaeopigments, but we expected such patterns, as over time, particles would sink slowly from the water column.

Enrichment resulted in a quick reduction of the total abundance (after 4 d), while the number of genera (i.e. richness) decreased only towards the end of the experiment (~30 d). This response was consistent with that found in other eutrophic estuaries (Netto & Valgas 2010, Armenteros et al. 2010). The response of nematodes was not consistent with the model proposed by Pearson & Rosenberg (1978). The model establishes that in high organically enriched sediments, the macrofauna is absent and nematodes are the dominant metazoans, predicting an initial in-

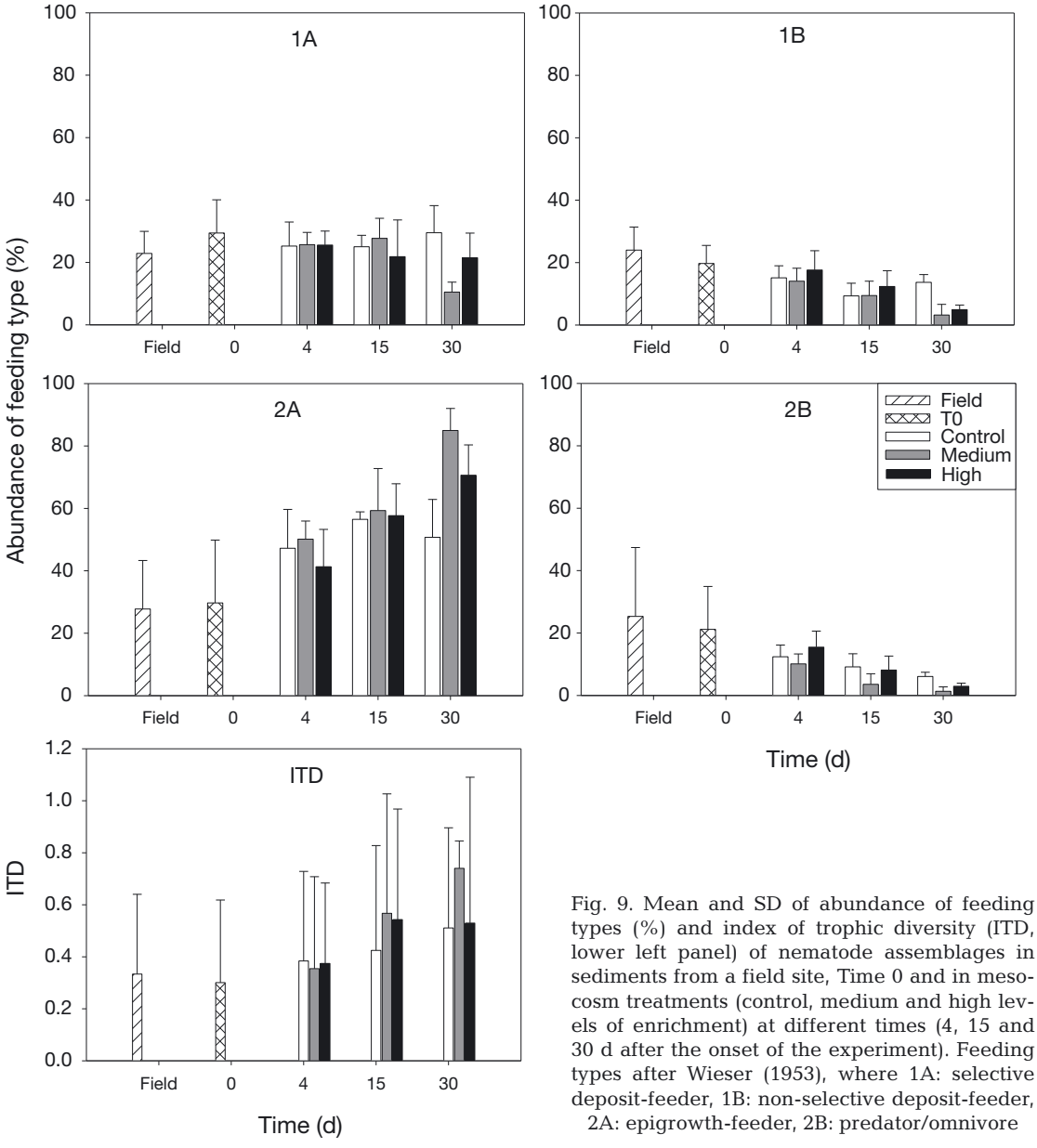

Fig. 9. Mean and SD of abundance of feeding types (%) and index of trophic diversity (ITD, lower left panel) of nematode assemblages in sediments from a field site, Time 0 and in mesocosm treatments (control, medium and high levels of enrichment) at different times (4, 15 and 30 d after the onset of the experiment). Feeding types after Wieser (1953), where 1A: selective deposit-feeder, 1B: non-selective deposit-feeder, 2A: epigrowth-feeder, 2B: predator/omnivore

crease in species richness in response to enrichment followed by a subsequent increase in abundance as richness starts to decline. The response of nematodes may perhaps fit with the dynamic equilibrium model, whereby richness peaks at intermediate levels of disturbance and productivity (Huston 1979). According to this model, a decrease in species richness means that few opportunistic species become overabundant. Dominance may increase either as a consequence of competitive exclusion or as a consequence of fewer species tolerating the harsh conditions.

Most of the genera were affected by enrichment, but some responses were difficult to interpret and may reflect non-linear or complex responses to the multiple environmental changes associated with enrichment (e.g. changes in dissolved oxygen and OM composition). For instance, *Anoplostoma*, which decreased in abundance in both enrichment treatments, has been reported as being favoured by organic enrichment (Kapusta et al. 2006). *Viscosia*, composed mainly of facultative predators able to exploit a wide range of food resources (Moens & Vincx 1997), had the lowest abundance at medium levels of enrichment and a higher abundance at the high level of enrichment. The increased density of *Pseudochromadora* in response to organic enrichment is more

logical: these are epistrate feeders that benefit from the availability and diversity of food resources (Pinto & Bemvenuti 2003, Kapusta et al. 2006). Some genera (*Oxystomina*, *Sabatieria*, *Terschellingia* and *Paradontophora*) appeared to be tolerant to enrichment, as they did not show changes among treatments. This is consistent with previous studies showing that such genera are well known for their proliferation in stressful conditions or in close association with sediment organic enrichment (Mirto et al. 2002). Species of the genus *Terschellingia* are tolerant to a diversity of stressors in soft bottoms (Schratzberger et al. 2006); *Sabatieria* and *Oxystomina* are tolerant to aquaculture deposition; and *Sabatieria* is well adapted to live in environments with high organic carbon loads, low oxygen and high sulfide concentrations (Jensen et al. 1992, Soetaert & Heip 1995). *Parodontophora* species have been reported to be unresponsive to changes in chl *a* sediment concentrations (Quang et al. 2016).

Enrichment also resulted in a reduction of the non-selective deposit-feeders (*Anoplostoma*, *Paralinhomoeus*) and predator/omnivores (*Viscosia*), which have faster metabolic rates, and presumably lower tolerance to hypoxia, than the epigrowth feeders (*Pseudochromadora*) and selective deposit-feeders (*Oxystomina*, *Terschellingia*) (Heip et al. 1985), which would make them less tolerant to the hypoxia. The decline of predators could also be a consequence of the loss of habitat complexity, as the higher abundance of predators indicates a more heterogeneous and well-structured trophic assemblage that might imply a higher habitat complexity (Semprucci et al. 2015). Metabolic rates, which are high in predators/omnivores but low in deposit and epigrowth feeders, should be a key factor explaining the responses: selective deposit-feeders showed only a temporary decrease and epigrowth feeders increased under conditions of enrichment. The combination of low metabolic rates and the feeding mode may thus enable tolerance or proliferation under enrichment. Selective deposit-feeders take advantage of the food supply (Armenteros et al. 2010) until the most deleterious effects derived from the organic input occurs. Epigrowth feeders, common in estuaries (Ndaro & Ólafsson 1999) may be able to exploit a diversity of food sources available after enrichment; *Pseudochromadora*, the dominant genus in this trophic group, consumes bacteria, microalgae and phytodetritus (Pinto & Bemvenuti 2003) and was clearly favoured by proteins and high values of BPC.

We found clear responses of trophic groups due to organic enrichment, despite the controversy of assigning whole genera to different trophic groups (Heip et al. 1985). This classification strategy ignores the complexity of nematode feeding habitats (Moens & Vincx 1997) and their trophic plasticity (Schratzberger et al. 2008). Most likely, the species composition and richness within each genus, and hence the likelihood of incorrectly assigning a specific organism to a particular trophic group, changes from site to site. It may well be that assemblages at Rocha Lagoon are dominated by a single species per genus which might drive the observed responses at the level of trophic groups. The effect of enrichment was also observed as a reduction in trophic richness (detected as an increase in the ITD index), which is contrary to with findings of other authors (Mirto et al. 2002, Alves et al. 2013) but consistent with Semprucci et al. (2013). Thus our study supports the hypothesis that enrichment alters nematode trophic structure. Nevertheless, we recognize the need to reevaluate the level of tolerance/sensitivity of the trophic groups to different stressors.

In contrast to the effects on trophic structure, enrichment did not seem to select for a particular life history (as quantified by the MI index), perhaps as a result of a high percentage of *K*-strategists (c-p value of 3). MI was initially proposed for the study of terrestrial and freshwater habitats (Bongers 1990), and marine and brackish ecosystems were included later (Bongers et al. 1991), but the lack of empirical evidence regarding life strategies of most marine genera resulted in a conservative use of this index. MI is responsive to river discharge and is more efficient than diversity indices in detecting effects of disturbance; however, it is also sensitive to sediment grain size (Semprucci et al. 2010, 2013). MI and c-p classes are sometimes unable to identify the dominant stressor when multiple stressors act together (Semprucci et al. 2013).

Given the multiple stressor nature of enrichment (OM content and quality are increased, but oxygen levels drop and drive the increases in concentrations of hydrogen sulfide and ammonia), we cannot identify which stressor drives the observed patterns in the nematode assemblages. Decreases in abundance may be driven mainly by hypoxia, as suggested by Gray et al. (2002) and Van Colen et al. (2009). Oxygen limitation is also suggested by the fact that the less responsive trophic groups were those characterized by low metabolic rates. Behavioural and physiological adaptations (e.g. migration to 'oxygen islands': Balsamo et al. 2012; slow movement and low metabolic rates: Warwick & Price 1979, Warwick & Gee 1984) may explain why some groups were not

affected in terms of their abundances (or they were able to maintain their densities). In addition, tolerance of hypoxia would have allowed greater access to OM in terms of amount and quality (availability of food), which are key controlling factors of the growth, metabolism and distribution of benthic communities within the substrate (Danovaro & Fabiano 1997, Venturini et al. 2011).

In summary, our study showed that organic enrichment can drive changes in the trophic status of sediments, reductions in the abundance and richness of nematodes, the loss of predators/omnivores and the dominance of selective deposit-feeders and epigrowth feeders. Our results also suggest that the study of nematode assemblages at the genus level is enough to detect effects of enrichment, consistent with other studies carried out elsewhere (Balsamo et al. 2012, Mirto et al. 2014), but also that the ITD seems to be a good candidate as an indicator of eutrophication effects on nematode assemblages.

Extrapolation from the experiment to nature should be done cautiously, since this is one of the main sources of misleading conclusions (Carpenter 1996). However, the responses of infauna to organic enrichment are governed primarily by the adaptations of species to conditions caused by organic load, thus extrapolation of responses from small-scale experiments to larger scales can be accepted (Zajac et al. 1998). Although our experimental set-up probably amplified the effects of treatments because of the stagnant conditions and the lack of water and sediment renewal, such amplification may be considered appropriate for a semi-enclosed coastal lagoon (Urban et al. 2009).

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