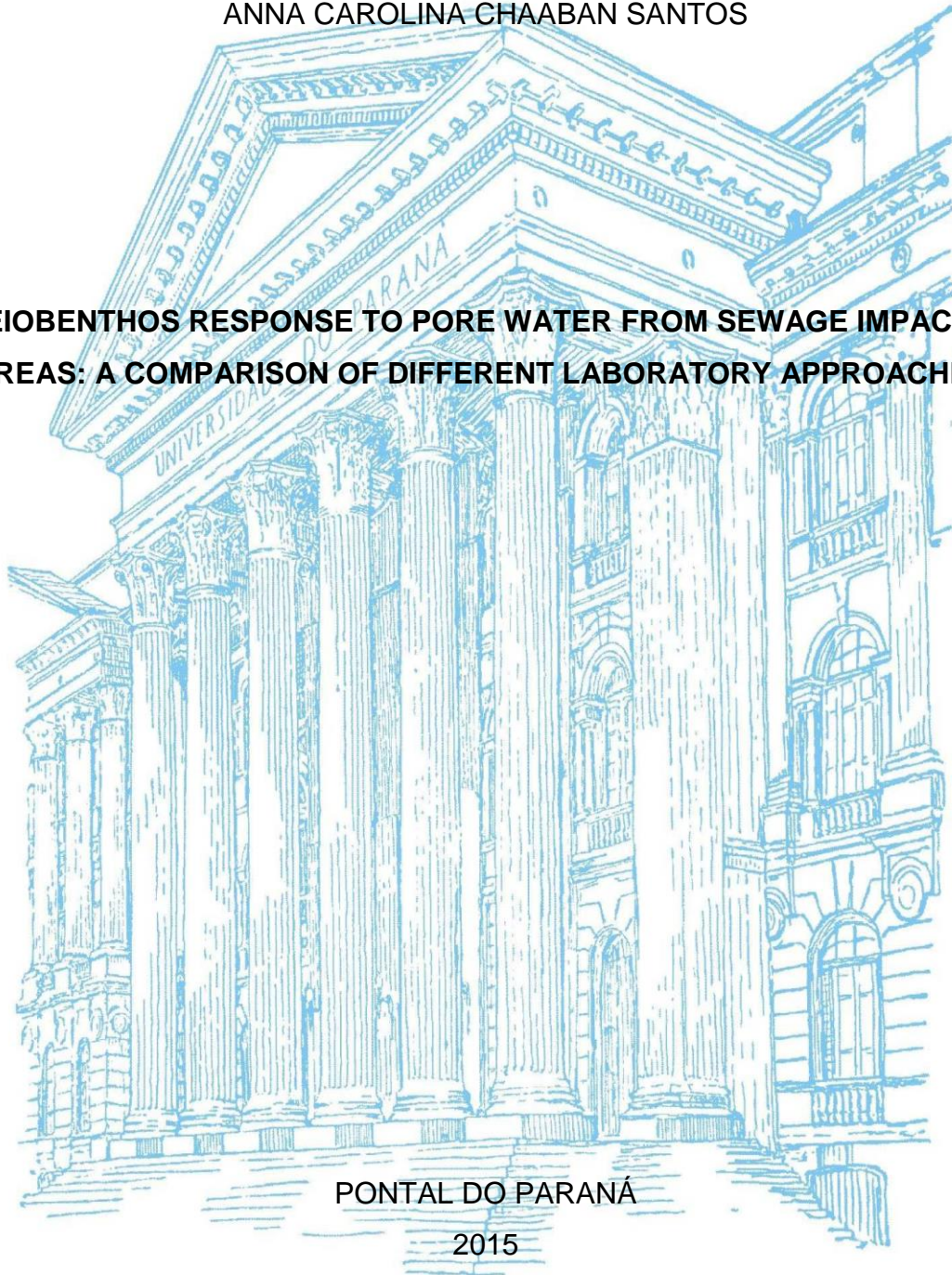


UNIVERSIDADE FEDERAL DO PARANÁ

ANNA CAROLINA CHAABAN SANTOS

**MEIOBENTHOS RESPONSE TO PORE WATER FROM SEWAGE IMPACTED
AREAS: A COMPARISON OF DIFFERENT LABORATORY APPROACHES.**



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Dissertação apresentada como requisito parcial para obtenção do grau de Mestre em Sistemas Costeiros e Oceânicos ao Programa de Pós Graduação em Sistemas Costeiros e Oceânicos, Centro de Estudos do Mar, Setor de Ciências da Terra, Universidade Federal do Paraná.
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**CURSO DE PÓS-GRADUAÇÃO EM SISTEMAS
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Ata da sessão pública de Defesa de Dissertação para obtenção do grau de Mestra em Sistemas Costeiros e Oceânicos, área de concentração Biologia e Ecologia de Sistemas. Ao primeiro do mês de abril de 2015, no anfiteatro do Centro de Estudos do Mar, às 14 horas, reuniu-se em sessão pública a Banca Examinadora da prova de Defesa de Dissertação da candidata ao Título de Mestra em Sistemas Costeiros e Oceânicos, **Anna Carolina Chaaban Santos**, composta pelos seguintes membros: Dra. Fabiane Gallucci (UNIFESP), como orientadora e presidente, Dr. Rodrigo Brasil Choueri (UNIFESP) e Dr. Paulo da Cunha Lana (UFPR/CEM), como examinadores. Após os esclarecimentos prestados pelo candidato às arguições feitas pelos membros da Banca, o Sr. Presidente suspendeu temporariamente a sessão a fim de que a Banca se reunisse em sessão secreta para deliberar sobre o resultado. Reaberta a sessão, o Sr. presidente deu conhecimento a candidata que, de conformidade com o Art. 67 da Resolução 65/09 CEPE, a dissertação foi aprovada, apenas condicionada à apresentação, no prazo de sessenta dias, da redação final com as alterações sugeridas.

Pontal do Paraná, 01 de abril de 2015.

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Orientadora e Presidente

Dr. Rodrigo Brasil Choueri (UNIFESP)
Membro Examinador

Dr. Paulo da Cunha Lana (UFPR/CEM)
Membro Examinador

Titulo da Dissertação: "*Meiobenthos response to pore water from sewage impacted areas: a comparison of different laboratory approaches.*"

Dedico esse trabalho a toda a minha família.

Aos meus pais e irmãos.

E ao Rafa, principalmente.

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"O que diferencia as pessoas extraordinárias das comuns:
amor, humor e inteligência" *Marcio Kühne*

RESUMO

As metodologias comumente utilizadas em avaliações de risco ecológico basearam-se, há décadas, em abordagens simplificadas. Embora essas abordagens tenham permitido o desenvolvimento de instrumentos regulatórios internacionais capazes de reduzir os efeitos adversos sobre os ecossistemas, elas geralmente sofrem de falta de realismo ecológico. Nesse contexto, os estudos de microcosmos que utilizam comunidades meiofaunais oferecem uma boa harmonização entre a complexidade do ecossistema e as configurações muitas vezes altamente artificiais de experimentos laboratoriais. Portanto, foi desenvolvido um experimento para investigar o potencial da abordagem do microcosmo utilizando a meiofauna como ferramenta para estudos ecotoxicológicos. O experimento testou simultaneamente os efeitos ecológicos da exposição à água intersticial sob influência de esgoto ao nível de comunidade, usando microcosmos de meiofauna, e ao nível de indivíduo, usando testes de fecundidade em laboratório com o copépode *Nitokra* sp. Especificamente, a experiência testou a toxicidade da água intersticial de três locais de acordo com um gradiente de contaminação. Ambas as abordagens foram eficientes na detecção de diferenças de toxicidade entre os locais mais limpos e os mais poluídos. No entanto, apenas os dados multivariados da análise de comunidade detectaram diferenças no gradiente de contaminação. Além das informações sobre toxicidade, os experimentos de microcosmos a nível de comunidade deram indicações sobre espécies sensíveis e tolerantes, efeitos ecológicos indiretos, bem como levantaram hipóteses sobre rotas de contaminação e biodisponibilidade a serem testadas. Os resultados deste estudo confirmam o potencial da abordagem com microcosmos usando comunidades naturais como uma ferramenta iminente para estudos ecotoxicológicos.

Palavras chave: meiofauna, nematódeos, *Nitokra* sp., microcosmos, água intersticial, ecotoxicologia, níveis de organização.

ABSTRACT

The usual procedures for ecological risk assessment have been based for decades on simplified approaches. Although these approaches allowed the development of international regulatory tools capable of reducing the adverse effects on ecosystems, they generally suffer from a lack of ecological realism. Within this context, microcosm studies using meiobenthic communities offer a good compromise between the complexity of the ecosystem and the often highly artificial settings of laboratory experiments. Therefore, an experiment was designed to investigate the potential of the microcosm approach using meiofauna as a tool for ecotoxicological studies. The experiment tested the ecological effects of exposure to sewage-impacted pore water simultaneously at the community level using meiofauna microcosms and at the individual level using laboratory fecundity tests with the copepod *Nitokra* sp. Specifically, the experiment tested the toxicity of pore water from three sites according to a contamination gradient. Both approaches were efficient in detecting differences in toxicity between the cleaner and most polluted sites. However, only multivariate data from community analysis detected differences in the gradient of contamination. In addition to information about toxicity, the community level microcosm experiments gave indications about sensitive and tolerant species, indirect ecological effects, as well as raised hypothesis about contamination routes and bioavailability to be tested. The results from this study confirm the potential of the microcosm approach using natural communities as an impending tool for ecotoxicological studies.

Keywords: meiobenthos, nematode, *Nitokra* sp., microcosm, pore water, ecotoxicology, levels of organization.

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Artigo Científico

Meiobenthos response to pore water from sewage impacted areas: a comparison of different laboratory approaches.

Resposta meiofaunal a água intersticial advinda de áreas impactadas por esgoto: uma comparação de diferentes abordagens de laboratório.

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1. INTRODUCTION

Marine ecotoxicological studies have traditionally used individual and population level parameters by means of single “indicator” species tests as a standard approach to predict the biological effects of a particular chemical and identify how dangerous it could be by showing a specific response (Chapman, 2002; Vighi and Villa, 2013). Studies at these levels of biological organization have the advantage of monitoring lethal and sublethal effects so that environmental damage may be controlled before causing dramatic consequences to the ecosystem (e.g. loss of species, disturbances on ecosystem functions). However, they have the disadvantages of being species specific and eventually the biological response of the selected model species won't necessarily promote a cascade effect through the upper levels of biological organization (Kimball and Levin, 1985; Vighi and Villa, 2013). In addition, single-species tests cannot account for the interactions and indirect effects that regulate the functioning of biological communities which makes the extrapolation to field conditions very difficult. Assuming that the aim of environmental protection is to preserve biologic communities and ecosystems, there is an urgent need to describe and predict effects of

contaminants at higher hierarchical levels accounting for the complexity of the ecosystems (SCENIHR, SCCS & SCHER, 2013; Vighi and Villa, 2013).

Microcosm tests using natural communities offer a good compromise between the complexity of the ecosystem and the often highly artificial settings of laboratory experiments (Rohr et al., 2016; Van den Brink et al., 2005). In addition to being more representative by considering the entire community with many species with different life traits and sensitivities, the microcosm approach preserves species interactions and considers the physico-chemical environment as well as different exposure routes. In this way, biotic and abiotic interactions typical of natural conditions are allowed to influence the outcome of the toxicity test that will be expressed in terms of community structure and function (Rohr et al., 2016). Therefore, community-based microcosm tests are usually more ecologically relevant than single-species tests and represent a potentially powerful tool for improving ecological realism of risk assessment (Cairns and Pratt, 1993; Chapman, 2002; Höss et al., 2014).

Marine sediments are sinks for large numbers and amounts of anthropogenic substances that enter the marine environment, which may exert toxic effects on the benthic biota. The use of the single-species toxicity tests is of particular concern in the assessment of the sedimentary environment which is exceedingly complex, and contains a high diversity of organisms. In addition, benthic organisms used in such tests are often restricted to specific habitats and not very representative in marine sediments. This is why the importance of integrating multiple lines of evidence in sediment quality assessments, including the benthos, has long been recognized (Carr et al., 1996; Chapman and Hollert, 2006; Choueri et al., 2010; Krull et al., 2014; Long and Chapman, 1985).

Within sedimentary environments, meiofauna communities comprise the most abundant and species-rich metazoans (Coomans, 2000; Heip et al., 1985; Lamshead and Boucher, 2003). They are ubiquitous in virtually all marine and estuarine environments and due to rapid generation time and fast metabolic rates, they play significant roles in the provision of energy to higher trophic levels. They also significantly contribute to important ecosystem functions such as decomposition and nutrient recycling processes (Bonaglia et al., 2014; Freckman, 1988; Hubas et al., 2010). Despite their ecological importance, this group represents an often neglected component of ecotoxicological studies. Intriguingly, however, is that meiofauna communities are particularly well-suited to microcosm studies. Due to the general lack of planktonic larvae, the manipulation and maintenance of natural communities in laboratory microcosms is relatively simple. Microcosms holding less than 200g of sediment typically contain many thousands of individuals and up to several dozen of species belonging to different trophic levels (e.g. bacterivores, microvores, herbivores, omnivores, and predators). In the last decades, the development of such microcosm setups

have allowed the investigation of the isolated effects of different pollutants in natural communities under controlled and reproducible conditions (Austen et al., 1994; Gallucci et al., 2015; Schratzberger et al., 2000).

The aim of the present study was to evaluate the suitability of using meiofauna community descriptors obtained in laboratory microcosms assays to assess marine sediment quality. This was done through the exposure of a meiobenthic natural community to sewage impacted pore-water and comparison of the responses of community structure with (i) effects observed by a single-species fecundity test with the meiobenthic copepod *Nitokra* sp. reared in the laboratory and (ii) sediment chemistry data. Adverse effects of sewage contaminated water have already been reported as a result from significant concentrations of pharmaceuticals and personal care products – PPCPs (Bila and Dezotti, 2003; Rodgers-Gray et al., 2000; Shareef et al., 2008), trace elements (Tjandraatmadja et al., 2008), surfactants (Jensen, 2004) and hydrocarbons (Jensen and Sverdrup, 2002). Therefore, we expected to find lower fecundity rates and changes in community structure as a response to pollutants present in the extracted pore water. Particularly for the community structure, we expected to observe a reduction in densities and diversity as well as the replacement of sensitive species by tolerant ones as a response to contamination (Pearson and Rosenberg, 1978). The experiment evaluated the response of the different endpoints in two different time periods. We expected to see direct toxic effects in the short-term (7 days) and a result of direct and indirect effects on the longer term (30 days).

2. METHODS

2.1. EXPERIMENTAL DESIGN

A laboratory experiment was designed to evaluate the effects of exposure to sewage-impacted pore water on the fecundity of the meiobenthic copepod *Nitokra* sp reared in the laboratory and on the structure of a meiobenthic community. Defaunated sediments from a reference site were individually contaminated with pore water from sediments collected at three sites located at different distances from the sewage outfall located at Araçá Bay, Brazil (Fig. 1). The sites were selected based on previous data from a field investigation (Gallucci et al., in prep.) that established regions affected by an inverse gradient of distance from the outfall, i.e., the further the more contaminated (within a radius of 150 meters), due to the major deposition at distant points. Sediments contaminated with pore water were used for microcosm set ups designed for the analysis of meiobenthic communities and for the set up of individual bioassays for the analysis of *Nitokra* sp. fecundity.

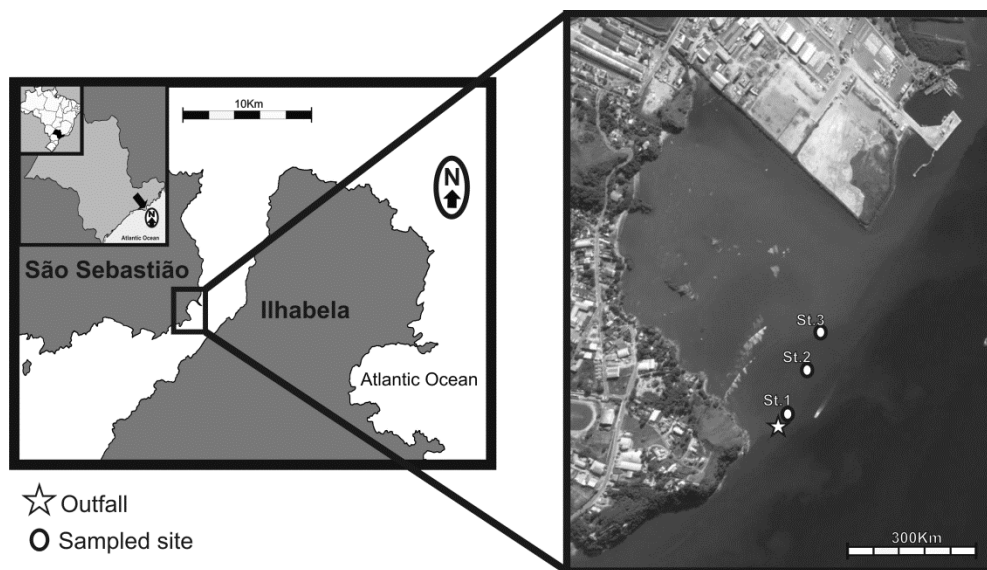


Figure 1. Map showing the location of the submarine outfall and sampling sites at Araçá Bay, São Sebastião, Brazil.

2.2. SEDIMENT COLLECTION AND DEFAUNATION

The sediments used in the microcosm experiment were collected at the shallow subtidal zone (ca. 1 m) of Cabelo Gordo beach, São Sebastião Channel, on the northern coast of São Paulo, southeastern Brazil (23° 49. 58' S, 45° 25. 31' W). This was considered as a reference site because it is a “zone of special management” in the marine spatial zoning of the *APA Marinha Litoral Norte* (North Shore Marine Protected Area). The surface sediment layers (top 5 cm) were collected using hand-corers (5 x 10 cm), placed in buckets and transported to the laboratory. After collection, sediment samples were defaunated by freezing for 12 hours and thawing at room temperature for 48 hours three times, according to Schratzberger et al. (2002). The defaunated sediment was stored until starting the experiments. A second sampling was performed 50 days later to obtain sediment containing meiofauna to set the exposure experiments (see item 2.4). Sediments containing meiofauna (first 5 cm layer), were transferred to boxes and gently mixed to ensure an even distribution of the meiofauna among microcosms.

2.3. EXTRACTION OF PORE WATER AND CONTAMINATION

The sediments used for the extraction of pore water were collected at the three sites (Fig.1) using a Van Veen grab sampler and were taken to the lab where they were gently homogenized and stored in plastic bags at a refrigerator (8 °C) until

extraction (approximately 3 hours after collection). The extraction was done by centrifugation of ca. 130 g of sediment at 400 rpm, for twenty minutes (Rachid, 2002) in a refrigerated room (20 °C). The process was repeated until the required amount (500 mL) was available. The extract obtained was kept in the refrigerator (8 °C) for a few hours. Redox potential and temperature were measured before and after each step and the variations followed the accepted standard for toxicity testing (Environmental Protection Agency, 2001). The contamination occurred through the saturation of the defaunated sediments with the extracted pore water, i.e. 35 mL of pore water for each 67 g of sediment.

2.4. MICROCOSM ESSAYS

2.4.1. Microcosm Set Up

Microcosms were set up with 4 cm of sediment layers in 500 mL Beakers (12 x 9 cm) by gentle mixing of spiked sediments with samples containing meiofauna. Afterwards, the Beakers were carefully filled with filtered seawater avoiding sediment suspension. The microcosms were constantly aerated and covered by parafilm to prevent evaporation and salinity increase. The Beakers were randomly assigned on the bench and maintained under dark conditions and 20°C, minimizing microalgal growth (Schratzberger et al., 2002) and photodegradation of any pollutants during 30 days.

2.4.2. Sampling Of Microcosms

Four replicates of each microcosm treatment (sites 1, 2 and 3) were randomly sampled at time intervals of 5, 15 and 30 days (T5, T15 and T30). Firstly, the overlying water was removed by siphoning and passed over a 45 µM mesh sieve to retain any organism in suspension. Next, the redox potential was measured at the sediment surface (ca. 1 cm depth) using an electrode connected to *Hanna Instruments HI 991003 meter*. Then, 3 cm³ of sediment were sampled for the analysis of chlorophyll *a* and pheopigments concentrations as a proxy of the microphytobenthos biomass and 2 cm³ was sampled for *Nitokra* sp. fecundity tests. The remaining sediment was fixed with formaldehyde 4 % for analysis of meiofauna (meiofaunal taxa and nematode morphospecies). Finally, sediments from one replicate microcosm from each contaminated treatment were sampled at the beginning (T0) and end (T30) of the experiment for determination of the concentrations of total ammonia, total

organic carbon, total nitrogen, phosphorus, arsenic, cadmium, lead, copper, chrome, mercury, nickel, zinc and surfactants.

2.4.3. Sample Processing

Meiofauna samples were washed through a 45 µM mesh sieve and extracted by flotation with Ludox TM 50 (specific density 1.18) (Heip et al., 1985). The retained material was stored in formaldehyde 4 % and stained with Rose Bengal. Meiofauna was counted and identified under a stereomicroscope. 10 % of the nematodes per microcosm were randomly picked, evaporated slowly in anhydrous glycerol and mounted on permanent slides for identification. A lowest and highest limit of 100 and 250 nematodes were established (i.e. when the total of nematodes was lower than 100, all individuals were picked for identification and when the total was higher than 250, only 250 individuals were taken). Nematodes were identified to genus level (Warwick et al., 1998) and further separated into morphospecies. Microphytobenthic samples were immediately frozen (-20 °C) and photosynthetic pigments extracted with 10 mL of 90 % acetone for 24 hours at a temperature of 4°C. Pigments were analyzed from absorbance spectra at 665 nm and 750 nm before and after acidification with HCL through a digital spectrophotometer. Concentrations of pigments were estimated using the equations of Lorenzen (1967).

The determination of chemical concentrations was done according to standard methods. For the quantification of arsenic, cadmium, lead, copper, chrome, nickel and zinc, sediments were acid-digested according to the method 3050B of Environmental Protection Agency (1996) and analyzed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (Environmental Protection Agency, 2007a). Mercury was tested by Cold-Vapor Technique (Environmental Protection Agency, 2007b), ammonia by selective electrode (Standard Methods Online, 1992), phosphorus with ascorbic-acid method, nonionic surfactants as cobalt thiocyanate active substances (CTAS) (Standard Methods, 2005), total nitrogen with determination by electrode ion selective and total organic carbon with oxidation of organic matter wet method with potassium dichromate in sulfuric acid medium (EMBRAPA, 1997). Spiked sample and blanks analyses were performed for the quality control of the chemical analyses. Recovery rates ranged from 80% to 120%.

2.5. *Nitokra* sp. ASSAY

Nitokra sp. bioassays were performed using 2 cm³ of the contaminated sediments just after contamination (T0), and after 15 and 30 days of incubation in meiofauna community microcosms (T15 and T30). The bioassay was conducted by adding 7,5 mL of water with adjusted salinity of 27 to 2 g of the sampled sediment. Toxicity was tested by adding 5 gravid

females to each replicate (modified from Lotufo and Abessa, 2002) Four replicates were done for each treatment (sites 1,2 and 3) and exposure period (T0, T15 and T30). The system was maintained at 25 °C \pm 2 temperature and a 16h: 8h light:dark photoperiod, during 7 days. Each sample was fixed with 1.5 mL of formaldehyde 10% and stained with Bengal Rose.

Samples were washed in a mesh of 45 micrometers to quantify the nauplii, copepodites, and adult females in a stereomicroscope. The fecundity rate of *Nitokra* sp. was determined through the reason $(N+C)/F$, where N, C and F are the number of nauplii, copepodites and adult females respectively. We have also performed a reference test to assess the organisms' sensitivity from a common batch, to assure that organisms would respond properly, not presenting a false positive result. Concentrations at 0.1; 1.0; 5.0; 10.0; 20.0 and 50.0 mg/L of potassium dichromate ($K_2Cr_2O_2$) were used as reference positive control for this test. Five gravid females were placed and counted daily for mortality until complete exposure time. The lethal concentration for 50% of the organisms after 96h exposure (LC_{50} - 96h) was determined and compared to the control chart of the laboratory.

2.6. STATISTICAL ANALYSIS

We used permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) to assess the significance of differences in faunal univariate measures. Meiobenthic abundance, nematode abundance, and copepod abundance were used as univariate indices. The analysis tested two factors: "Treatment" (3 levels: Sites 1, 2 and 3) and "Exposure period" (5, 15 and 30 days) as fixed factors. The same factors were used to assess significant differences in multivariate structure of nematode assemblages. We also used PERMANOVA with "Treatment" (3 levels: Sites 1, 2 and 3) and "Exposure period" (3 levels: 0, 15 and 30 days) as fixed factors to assess the significance of differences in the *Nitokra* sp. bioassays. PERMANOVA was used instead of the traditional ANOVA after testing for the homogeneity of variances (Cochran's test) of all variables and obtaining significant p-values ($p < 0.05$) even after transformation. Although PERMANOVA was designed for multivariate analysis on distance matrices, it can be used for univariate ANOVA, and because it calculates p-values using permutations rather than relying on tabulated p-values, it does not have to meet the traditional ANOVA assumptions (Anderson et al., 2008). Particularly regarding the homogeneity of variances between groups, PERMANOVA is largely unaffected by heterogeneity for balanced designs (Anderson and Walsh, 2013). To visualize the multivariate structure of the meiofauna and nematode assemblages, non-metric multi-dimensional scaling ordination (nMDS) (Clarke and Ainsworth, 1993) was performed based on the same matrix used for the PERMANOVA. PERMANOVA tests were conducted

on Euclidean distance matrices, which is recommended for microcosm experiments because as a closed system it does not have the income of new species and because our hypothesis considers that some species can disappear due to the toxicity (Anderson et al., 2011). Pairwise *a posteriori* multiple comparisons tests were performed when significant differences were detected ($p < 0.05$). The residuals were permuted using unrestricted permutation of raw data.

Similarity percentages programme (SIMPER) was used to determine the contribution of individual morphospecies to the average dissimilarity between treatments and/ or exposure period. To check if there were any specific feeding types of nematodes affected by the exposure to contaminated sediments, nematode species were assigned to feeding types according to Wieser's (1953) classification based on the morphology of the buccal cavity: selective deposit feeders (1A), non-selective deposit feeders (1B), epigrowth feeders (2A) and predator/omnivores (2B) and the percentage of contribution of each feeding type to the total abundance of nematodes was calculated for each sample.

The associations among the variables measured in the current study were assessed by factor analysis with principal component analysis as the extraction method (FA/PCA) after varimax normalised rotation of the data. Data of descriptors of meiofauna community (total abundance of nematodes and copepods, nematode species richness (S), evenness (J') and Shannon-Wiener diversity (H') and abundances of the taxa *Sabatieria* sp.1, *Oncholaimellus* sp.1, *Pseudosteineria* sp.1, *Nudora* sp.1 and *Dichromadora* sp.1), *Nitokra* sp. fecundity, microphytobenthic parameters (concentrations of chlorophyll *a* and pheopigments), and sediment chemical parameters (redox potential, total organic carbon contents, levels of total ammonia and nitrogen, As, Cd, Cu, Cr, Hg, Ni, Pb, Zn and LAS) measured at the beginning and at the end of the microcosm assays were assessed for multivariate associations. The variables were autoscaled (standardised) so as to be treated with equal importance. Only factors accounting for at least 10% of explanation of the total variance were analysed. The loading cut-off was set at |0.38|. The relevance of the observed associations to each of the 24 cases was estimated by calculating the factor score from each case for the centroid of all cases for the original data.

All univariate and multivariate analysis were performed using the software PRIMER® (version 6) (Clarke and Gorley, 2006) with the additional add-on package PERMANOVA (Anderson et al., 2008) and STATISTICA® (version 12StatSoft).

3. RESULTS

3.1. ENVIRONMENTAL DATA

Chlorophyll-*a* and pheopigment concentrations varied from 0.305 to 0.556 $\mu\text{g}\cdot\text{cm}^{-3}$ and from 0.04 to 0.25 $\mu\text{g}\cdot\text{cm}^{-3}$, respectively. There were no significant differences between treatments or exposure periods (PERMANOVA $p>0.05$). Redox potential measures varied from -34mV to 53mV and differed between treatments depending on the time of exposure (significant interaction effect between “exposure period” and “treatment”). Those differences were, nevertheless, not associated to meiofauna descriptors and *Nitokra* fecundity (item 3.4). After 5 days of exposure all stations differed significantly from each other: St. 1 showed lower redox values and St. 3 exhibited higher values while Station 2 was in an intermediary position. Within 15 days of exposure, Station 1 differed significantly, showing higher redox values than St. 2 and 3 and within 30 days, there were no differences between the treatments. Total ammonia and organic carbon increased in all stations over the time of exposure.

Station 1 showed the lowest concentrations of metals (except for mercury) and surfactants when compared to stations 2 and 3, although the differences were small among all stations (Table 1). In general, levels of metals and As were low in all stations, with few exceptions. Cd levels exceeded the Threshold Effects Level (TEL) set by CCME (2001) at all stations and Hg was equal to TEL at station 1. In comparison to sediment quality guidelines developed by Brazilian Southeastern estuaries (Paranaguá and Santos esturine systems) (Choueri et al., 2009), besides Cd and Hg exceedences (in agreement to the CCME (2001) guidelines), As (at all stations) and Ni (at station 3) were found at moderate levels. However, as it will be discussed further, only Cd and Ni were negatively associated to meiofauna community descriptors and *Nitokra* sp. fecundity (item 3.4).

Table 1. Concentrations of metals, phosphorus, total nitrogen (TN), total ammoniacal nitrogen (TAN), total organic carbon (TOC) and surfactants (Surf.) in the interstitial water extracted from sediments contaminated with interstitial water from St. 1, St.2 and St.3 at T0 and T30.

Treatment	Time	As	Cd	Pb	Cu	Cr	Hg	Ni	Zn	TN	TAN	TOC	Surf.
St. 1	T0	4.2	0.59	3	1.6	12	0.13	3.2	21	20	4.6	0.12	1
St.2	T0	4.3	0.59	3	1.4	12	0.12	3	22	20	6	0.05	1.3
St.3	T0	4	0.6	3.3	1.9	13	0.12	3.7	23	20	6.4	0.05	1.3
St. 1	T30	3.3	1	3.2	1.8	12	0.12	3.9	23	42	15	0.33	1.2
St.2	T30	4.3	1.1	3.8	1.8	12	0.12	4	24	20	21.1	0.25	1.3
St.3	T30	4	1.1	3.2	1.8	13	0.11	4	25	20	17.3	0.33	1.3

3.2. MEIOFAUNA COMMUNITY

The meiofauna was represented by 9 higher taxa with densities ranging from 38 to 2501 individuals per microcosm. Nematodes numerically dominated all samples, representing from 60 to 100% of the total metazoan meiofauna per microcosm, and exhibited a total of 49 morphospecies belonging to 41 genera. Densities ranged from 28 to 2350 individuals per microcosm. Copepods were the second most abundant group (0 to 33% of total meiofauna) with densities ranging from 0 to 278 individuals per microcosm.

Results of the two-way PERMANOVA showed significant differences for nematode and copepod abundances between treatments, independently of the duration of exposure (Table 2). In accordance to the pollution gradient, abundances of both groups decreased from site 1 towards sites 2 and 3 (Fig. 2). Post-hoc analysis confirmed significantly lower nematode abundances at sites 2 and 3 when compared to site 1. Copepod abundances did not differ between sites 1 and 2, and were significantly lower at site 3. Nematode species richness and diversity did not differ between treatments (Table 2). Nematode and copepod abundances and nematode species richness also decreased over time, being significantly lower after 15 days of exposure, independently of the treatment (Table 2, Fig. 3). Nematode densities and species richness remained significantly lower after 30 days of exposure when compared to 5 days (Fig. 3).

As for the univariate measures, PERMANOVA analysis for the multivariate structure of nematode assemblages revealed significant differences in assemblage structure between treatments and between exposure periods, with no significant interaction between the two factors (Table 3). Post-hoc analysis revealed significant differences between the three sites and the two exposure periods. Figure 4 shows the MDS ordination for nematode assemblage structure for treatments. Although there is not a clear distinction between sites, there was a greater overlap between Sts 1 and 2 with a greater separation of St. 3. Indeed, SIMPER analysis revealed a greater distance between Sites 1 and 3 (average distance: 197129) with St. 2 at an intermediate position (average distances of St. 2 with St. 1 and 3: 148867,68 and 76696,12, respectively), confirming a pattern that agrees with the pollution gradient. According to SIMPER analysis, differences between treatments were mainly due to lower densities of the most abundant species (i.e., *Nudora* sp. 1, *Pseudosteineria* sp. 1 and *Dichromadora* sp.1) in St. 2 and 3 when compared to St. 1, with higher magnitude of differences in average abundances at St. 3. It also pointed for the lower abundances of *Oncholaimellus* sp.1, a predator who was not particularly abundant, in Sts 2 and 3 compared to St 1. Station 3 also differed from both St. 1 and 2 by showing an increase in the average abundance of the species *Sabatieria* sp.1.

There were also significant differences in nematode assemblage structure between exposure periods independently of the treatment (Table 3). Assemblages were significantly

different at both 15 and 30 days of exposure when compared to day 5. Simper analysis indicated that differences were due to lower abundances of the most abundant species (i.e., *Nudora* sp. 1, *Pseudosteineria* sp. 1 and *Dichromadora* sp.1) after 15 and 30 days.

In this study, all feeding types of nematodes (1A: selective deposit feeders, 1B: non-selective deposit feeders, 2A: epigrowth feeders and 2B: predator/omnivores) were represented in all treatments. Significant differences between treatments were detected for the densities of the groups of epigrowth feeders (2A) and predators (2B) (Table 4), which were both significantly lower in Sts 2 and 3 compared to St. 1.

3.3. *Nitokra* sp. FECUNDITY TEST

Similar to meiofauna measures in the microcosm setup, *Nitokra* sp. fecundity showed significant differences between treatments and between exposure periods, with no interaction between the two factors (Table 2). As for nematodes, number of nauplii and copepodite per female was significantly lower at sites 2 and 3 compared to site 1, independently of exposure period (Fig. 2). Also in agreement with meiofauna results, toxicity as measured by *Nitokra* sp. fecundity was significantly higher (i.e. fecundity significantly lower) after 15 days of contamination (Fig. 3). Results from the reference test were considered acceptable (i.e. within the limits of the control chart of the laboratory (2x the standard deviation), with a $LC_{50} - 96h$ of 13.89 mg/L of potassium dichromate.

Table 2. Results from two-way PERMANOVA analysis for meiobenthic univariate indices and *Nitokra* sp. fecundity. EP=Exposure period and Treat = Treatment. Values in bold represent $p < 0.05$.

	<i>df</i>	<i>MS</i>	<i>Pseudo-F</i>	<i>P(perm)</i>	<i>Unique perms</i>
<i>Nematode density</i>					
Treatment	2	1,224E6	7,7314	0,004	999
Exposure period	2	1,7756E6	11,212	0,001	997
EP x Treat	4	1,3139E5	0,82971	0,538	998
Residue	27	1,5836E5			
Total	35				
<i>Copepod density</i>					
Treatment	2	25489	8,9419	0,002	998
Exposure period	2	13235	4,643	0,018	997
EP x Treat	4	4083,8	1,4327	0,231	999
Residue	27	2850,5			
Total	35				
<i>Nitokra sp.fecundity</i>					
Treatment	2	166,55	3,4406	0,046	998
Exposure period	2	453,99	9,3783	0,002	998
EP x Treat	4	68,104	1,4069	0,258	999
Residue	27	48,408			
Total	35				
<i>Nº of nematode species</i>					
Treatment	2	27,444	3,0811	0,068	997
Exposure period	2	101,69	11,417	0,001	999
EP x Treat	4	9,6944	1,0884	0,377	998
Residue	27	8,9074			
Total	35				

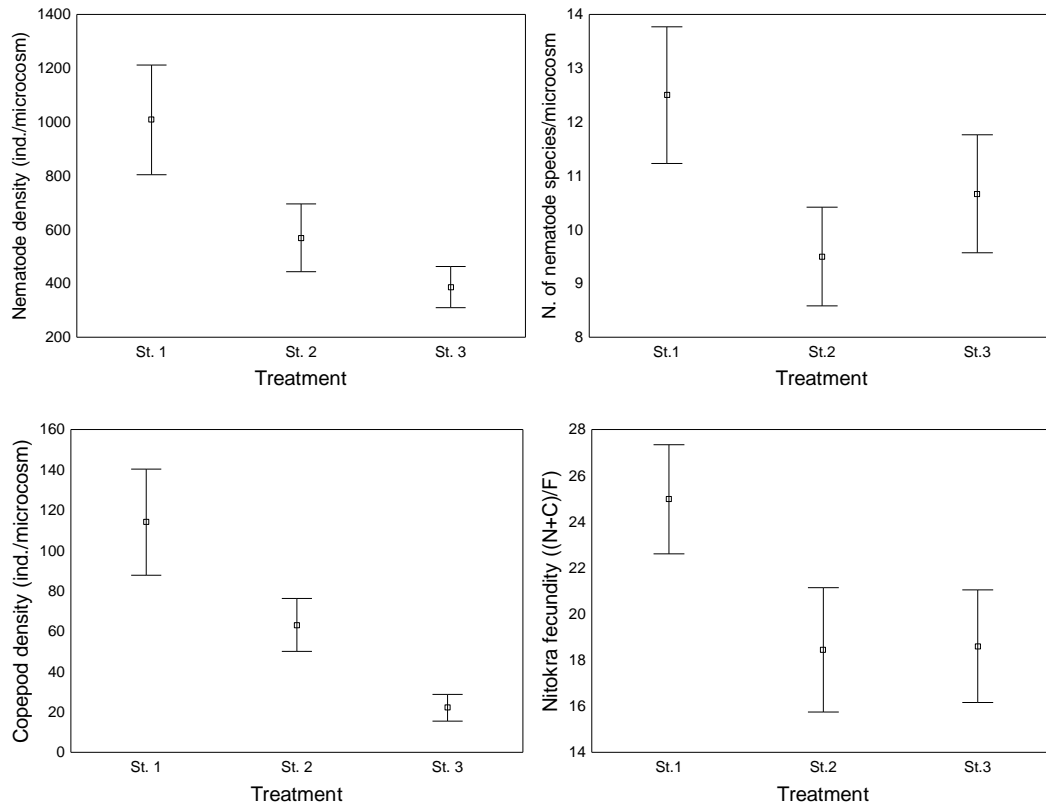


Figure 2. Mean and standard error of nematode and copepod abundances, nematode species richness and number of nauplii + copepodite per female ((N+C)/F) in the different treatments.

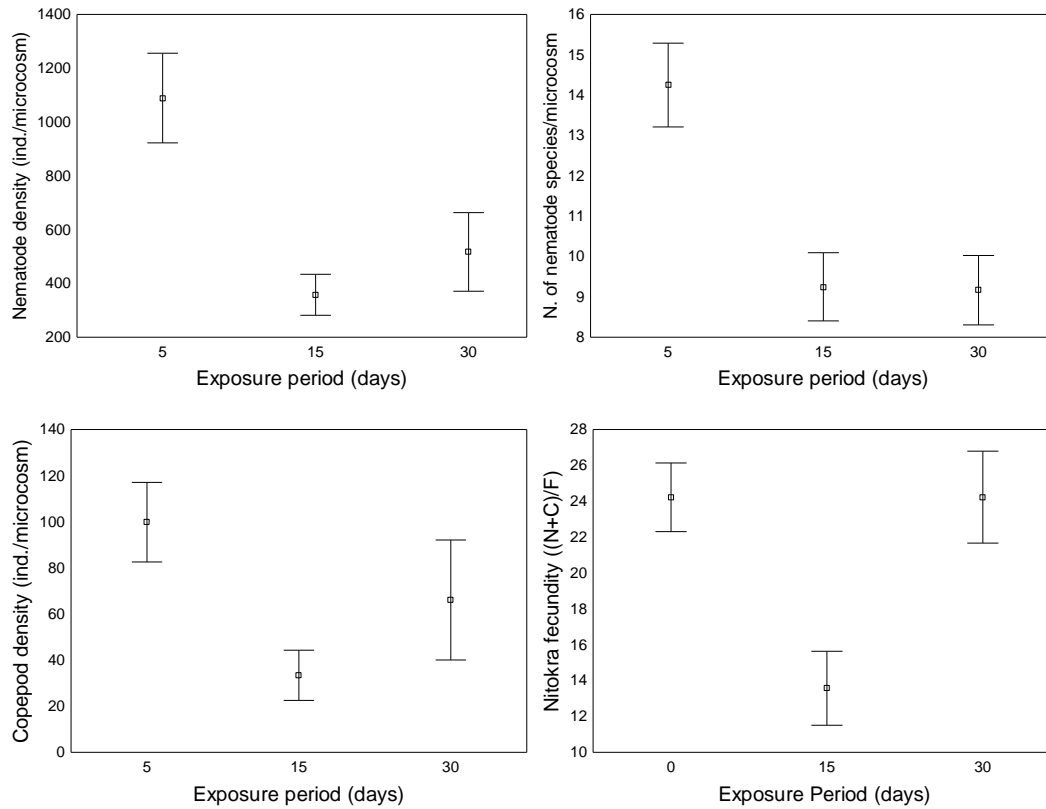


Figure 3. Mean and standard error of nematode and copepod abundances, nematode species richness and number of nauplii + copepodite per female (N+C)/F in the different exposure periods.

Table 3. Results of PERMANOVA and post-hoc for differences in the multivariate structure of nematode assemblages. EP=Exposure period and Treat = Treatment. Values in bold represent $p < 0.05$.

	<i>df</i>	<i>MS</i>	<i>Pseudo-F</i>	<i>P(perm)</i>	<i>Unique</i>
Community					
Treatment	2	3,0207E5	8,0805	0,001	999
Exposure period	2	2,2861E5	6,1153	0,003	999
EP x Treat	4	39344	1,0525	0,409	998
Residue	27	37383			
Total	35				
<hr/>					
	<i>t</i>	<i>P (perm)</i>			
Treatment					
St. 1 vs. 2	1,9566	0,046			
St. 1 vs. 3	3,6783	0,001			
St. 2 vs. 3	2,569	0,002			
<hr/>					
Exposure Period					
5 x 15 days	3,4726	0,002			
5 x 30 days	2,298	0,012			
15 x 30 days	0,96717	0,383			

Table 4. Results of PERMANOVA for feeding types of nematode assemblages. EP=Exposure period and Treat = Treatment. Values in bold represent $p < 0.05$.

	<i>df</i>	<i>MS</i>	<i>Pseudo-F</i>	<i>P(perm)</i>	<i>Unique perms</i>
1A					
Treatment	2	23,014	1,376	0,282	998
Exposure period	2	45,583	2,7255	0,092	999
EP x Treat	4	15,599	0,93266	0,459	998
Residue	27	16,725			
Total	35				
1B					
Treatment	2	1,3304E5	2,9904	0,078	999
Exposure period	2	3,932E5	8,8383	0,003	999
EP x Treat	4	20310	0,45651	0,783	999
Residue	27	44489			
Total	35				
2A					
Treatment	2	4,3068E5	15,59	0,001	999
Exposure period	2	2,3193E5	8,3957	0,002	999
EP x Treat	4	31191	1,1291	0,369	999
Residue	27	27625			
Total	35				
2B					
Treatment	2	11948	5,2152	0,011	998
Exposure period	2	50655	22,11	0,001	999
EP x Treat	4	3951,3	1,7247	0,181	998
Residue	27	2291			
Total	35				

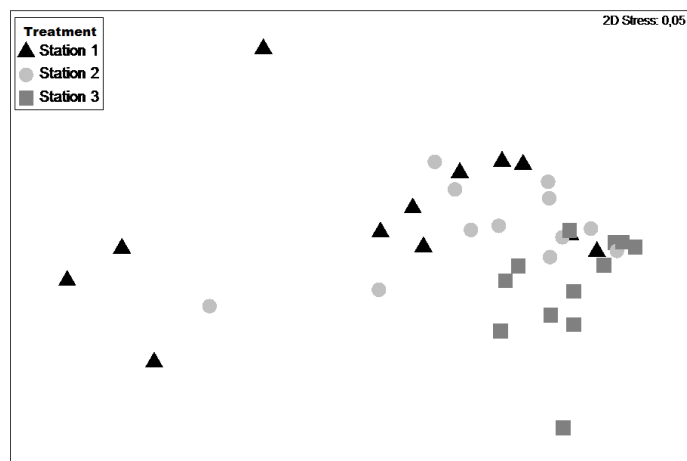


Figure 4. nMDS ordination from abundances of nematode species in the different sites.

3.4. MULTIVARIATE RELATIONSHIPS BETWEEN VARIABLES

Three factors were extracted from the original dataset which together accounted for 69.3% of the total explained variance (Table 5). The first factor (F1, which explained 41.69% of the variance) showed meiobenthic community descriptors (S, total abundance of nematodes and copepods, abundances of *Oncholaimellus* sp.1, *Nudora* sp.1, *Dichromadora* sp.1 and *Pseudosteineria* sp.1) and *Nitokra* sp. fecundity negatively associated with levels of chemicals in the sediments (total ammonia, LAS, and all metals except Hg). The second factor (F2, which contributed with 16.8% of the explained variance) showed the association of levels of pheopigments, total ammonia, total nitrogen, TOC, and some metals (Cd, Cu, Ni and Zn). This group was negatively associated with potential redox and As concentrations. The third factor (F3, which explained 10.95% of the total variance) showed the association of S, J', H', abundance of *Sabatieria* sp.1 and pheopigment concentrations. Levels of chlorophyll *a* were negatively associated with these variables.

Factor scores are shown in Fig. 5. F1 scores exhibited notable differences among treatments (sites). Site1 showed negative scores at both T1 and T3 for almost all replicates (except one replicate showing score close to 0), site 2 showed negative scores at T1 and positive scores at T3, and site 3 showed positive scores for F1 both at T1 and T3. F2 scores, in turn, showed remarkable differences between times of exposure. Sites 1, 2, and 3 showed positive scores for F2 at T1 and negative scores at T3. The scores estimated for F3 showed some differences among replicates within the same treatments. High positive scores for F3 were estimated for site 3 consistently at T1, and also for some replicates of site 1 both at T1 and at T3. Negative scores for F3 were estimated consistently for site 2 at T1, but the lowest values were estimated for some replicates of site 3 at T3.

Table 5. Factor loadings after varimax rotation for the three factors obtained in the FA/PCA. The explained variance of each factor is given in percentage of the total variance in the original data matrix. Marked loadings are $\geq|0.38|$

Variable	Factor Loadings		
	Factor 1	Factor 2	Factor 3
Species richness	-0,58	0,32	0,62
Equitativity (J')	-0,19	0,18	0,74
Diversity (H')	-0,36	0,28	0,79
Nematoda (N)	-0,86	0,12	0,37
Sabatieria sp.1	0,14	0,25	0,65
Oncholaimellus sp.1	-0,84	0,37	0,14
Nudora sp.1	-0,90	0,03	0,14
Dichromadora sp.1	-0,85	0,08	0,19
Pseudosteineria sp.1	-0,79	-0,01	0,20
Copepoda(N)	-0,82	-0,17	0,29
<i>Nitokrasp.</i> fecundity	-0,38	-0,15	-0,06
Chlorophyll	0,12	0,29	-0,65
Pheopigments	-0,09	-0,56	0,38
Redox	0,11	0,77	0,37
Total ammonia	0,49	-0,75	-0,20
Total nitrogen	-0,28	-0,68	-0,10
Total organic carbon	0,20	-0,92	-0,27
As	0,09	0,66	0,02
Cd	0,43	-0,82	-0,24
Cu	0,60	-0,51	0,34
Cr	0,69	0,15	0,18
Hg	-0,72	0,24	0,28
Ni	0,63	-0,74	0,06
Pb	0,56	-0,38	0,16
Zn	0,80	-0,47	-0,14
Surfactants	0,77	0,17	-0,11
Explained variance	41.92%	16.84%	10.53%

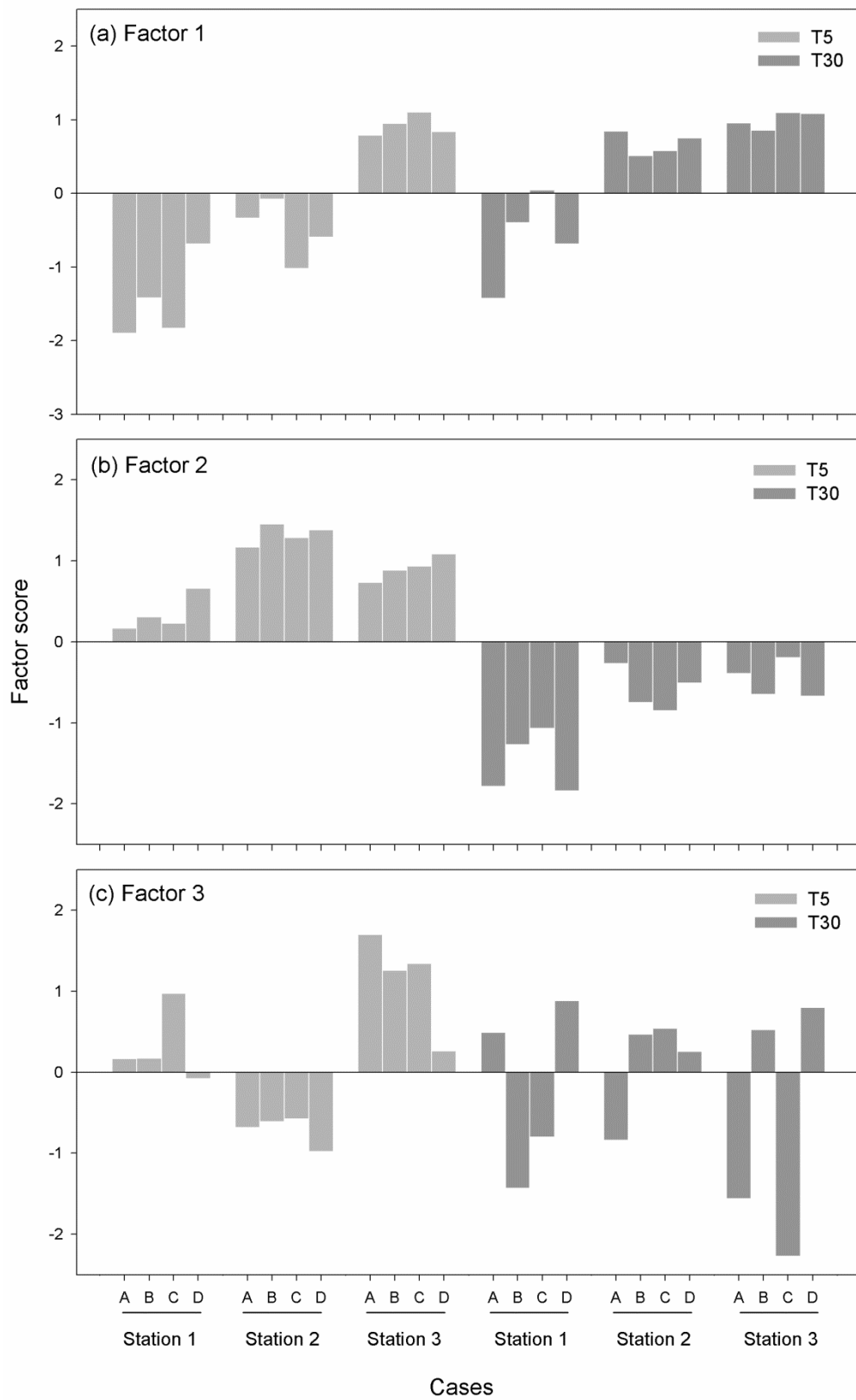


Figure 5. Factor loadings after varimax rotation for the three factors obtained in the FA/PCA.

4. DISCUSSION

Both *Nitokra* sp. and meiobenthic community bioassays detected significant negative effects in treatments St.2 and St.3 when compared to St. 1, which presented better environmental quality conditions. Indeed, multivariate relationships between variables indicated a clear relationship between *Nitokra* sp. fecundity and meiofauna community parameters (Factor 1). Both endpoints were negatively related with chemical concentrations indicating an effect of the contaminant sources on both test-organisms. Since only a few contaminants are apparently contributing to the observed effects, i.e. associated with effects in the FA/PCA and/or at a concentration above the threshold effects level (TEL) (CCME, 2001) or Brazilian sediment quality guidelines (Choueri et al., 2009), the observed association of contaminants levels and individual and ecological responses may have a contribution of other unmeasured substances present in sewage (e.g. Bao et al., 2013; Gyedu-Ababio and Baird, 2006; Todd et al., 2010). Toxicity was already reported as a result from environmental relevant concentrations of pharmaceutical compounds (Bila and Dezotti, 2003; Shareef et al., 2008; Maranhão et al., 2014), personal care products (Cortez et al., 2012) and hydrocarbons (Jensen and Sverdrup, 2002), for example, among other sewage components. In addition, interactive effects between metals (Hagopian-Schlekat et al., 2001), and metals and other potentially toxic substances in sediment, such as ammonia (Campos et al., 2016), can not be ruled out.

Whereas *Nitokra* sp. fecundity was similarly affected at St. 2 and St.3 compared to St. 1, analysis of nematode and copepod densities exhibited a conspicuous declining gradient, indicating the treatment St.3 as the most impacted. Accordingly, factor analysis exhibited more evident toxic effects at site 3, which showed positive scores (i.e. lower values for the biological parameters and higher values for chemical concentrations) for factor 1 already after 5 days of exposure maintaining these positive values after 30 days. Station 2 also showed positive score values, but only after 30 days of exposure. Site 1, on the other hand, showed better environmental conditions after both 5 and 30 days of exposure since it showed higher values for the biological parameters and lower values for contaminant concentrations. These results agree with the inverse gradient of increasing contamination with distance from the outfall observed in the field. Such inverse gradient indicates that precipitation of sewage particles and associated contaminants mainly occur when sewage is mixed with sea water, a phenomenon known as the "salting out effect", which is the decrease of solubility of organic compounds due to the increase of salinity, providing greater precipitation (Qi et al., 2008). Metals may also coagulate, flocculate and precipitate as salinity increases (Chapman and Wang, 2001).

Multivariate community data showed higher differences between treatments St.1 and St.3, with St. 2 at an intermediate position between the two, further confirming the pollution gradient. These changes were mainly a result of the decrease in densities of the most abundant species already after 5 days of exposure, as indicated by the lack of interaction between the factors “treatment” and “exposure period” for both univariate and multivariate analysis. This short-term decrease in densities suggests mortality, rather than other sublethal effects, as a cause. Although mortality of the most abundant groups indicates a non species-specific response, we have also detected a differential response of predacious nematodes as a group and SIMPER analysis also indicated lower average abundances of the predator nematode *Oncholaimellus* sp. 1 at Sts 2 and 3 compared to St. 1. Such results are particularly interesting given that the same community has shown a similar response to contamination of sediments by antifouling biocides (Gallucci et al. 2015). This corroborates the group of predacious/omnivorous nematodes as more sensitive to disturbance (Bongers et al., 2001), and also confirms the genus *Oncholaimellus* as a sensitive genus (Danovaro et al., 1995; Gallucci et al. 2015). Whereas the reasons for higher sensitivity of predacious nematodes to disturbance might be due to their longer-lived life strategy (Bongers et al., 2001), we believe that regarding contamination, the short-term response directed to this functional group might be due to their smooth cuticle, which is more permeable than more ornamented cuticles (Fonseca and Fehlaue-Ale, 2012), therefore being a major route for uptake of contaminants (Howell, 1983; Kammenga et al., 1994).

In addition to mortality of some nematode populations, we have also observed the increase in densities of the opportunistic species *Sabatieria* sp.1. at treatment St.3. The increase in *Sabatieria* sp. 1 density is probably an indirect effect of contamination which has caused the death of abundant species living space/resource available for the opportunistic species to flourish. This genus is known as having a direct positive relationship with anthropogenic disturbance (Schratzberger et al., 2009), being highly adapted to fit into new habitats, conditions or resources (Heip et al., 1985) and an indicator of a poor ecological quality status due to its tolerance to pollution (Austen and Somerfield, 1997; Moreno et al., 2011; Soetaert et al., 1995). F3 in the multivariate analysis further illustrates possible indirect effects of contamination. Loadings for F3 showed a positive relationship between species richness, diversity, equitability and densities of *Sabatieria* sp. The factor scores showed that these values were particularly high for the treatment St.3. This result indicates that the lower dominance of the typically most abundant species in this treatment has indirectly caused an increase in diversity and equitability indices, at the same time it has allowed the increase in abundance of a tolerant and opportunistic species.

Concerning the exposure period, there was a decrease in nematode diversity and abundance after 15 days, regardless of treatment. Although this could be an effect of the enclosure in the microcosm experiment (Gallucci et al., 2015; Schratzberger et al., 2002), it is important to note that *Nitokra* sp. fecundity test also showed significantly lower fecundity rates in sediments which had been exposed for 15 days, independently of the treatment. Such results suggest that toxicity has increased during the exposure period and lower meiofauna densities and diversity are probably a result of this increased toxicity in all treatments rather than a microcosm effect.

The increased toxicity exhibited by the sediments at 15-day exposure reinforces that unmeasured chemicals, as the PPCPs, may be the cause of the observed toxicity. It is known that some organic pollutants, including PPCPs, are degraded into substances with higher toxicity than the parent compound. Köhler et al. (2006) observed increased ecotoxicity of an industrial wastewater following a reduction in the organic pollutants load after biological treatment, suggesting a transformation of the parental pollutant into more toxic by-products. Regarding PPCPs, Triclosan, which was showed to exert toxicity effects at environmentally relevant concentrations as the parental compound (Cortez et al., 2012), is also converted by photodegradation into toxic substances such as dioxins and furans (Kanetoshi et al., 1987; Latch et al., 2003). Other pharmaceuticals compounds which are known to produce metabolites that may be toxic in the environment include the antibiotics cefazolin and cephapirin (Wang and Lin, 2012), acetylsalicylic acid, carbamazepine, diclofenac, sulfonamides, among others (Celiz et al., 2009).

The integration of biotic and environmental data also showed, in factor 2, a clear separation of the exposure periods. After 30 days of exposure, redox values and ammonia concentrations were generally higher when compared to 5 days. This was true for sites 1, 2 and 3 which showed negative scores at 5 days and positive scores after 30 days. This is probably a result of microbial degradation processes and accumulated nitrogen excretion by the test-organisms in the microcosms. Despite the potential of those changes to affect the test-organisms and the outcome of the test, changes were similar to all treatments excluding the possibility of these being an indirect effect of the contamination.

5. CONCLUSION

Both single-species test and community level microcosm approaches agreed in detecting differences in toxicity between the cleaner and the potentially most polluted sites. However, only data derived from community analysis detected the expected differences in the gradient of contamination. These results indicate that the microcosm approach using natural communities was more sensitive to the detection of more subtle impacts when

compared to the single-species fecundity approach. In addition to information about toxicity, the community level microcosm experiments gave also indications about sensitive and tolerant species, indirect ecological effects, as well as raised hypothesis about contamination routes to be tested. These results confirm that analysis at community level are generally more refined by giving extra information compared to the traditional single species level approach (Chapman, 2002; Clements, 2000; Höss et al., 2014). Considering the importance of free-living marine nematodes for benthic ecosystems, the microcosm approach using natural meiobenthic communities might be a valuable addition as a higher tier approach in ecological risk assessment, providing highly relevant ecological information on the toxicity of contaminated sediments.

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