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Effects of Endosulfan (Thiodan 35 EC®) on Meiofauna Community Through a Microcosm Approach

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ABSTRACT

A microcosm experiment with natural community of meiofauna was performed to test the effects of the organochlorine pesticide Thiodan 35 EC (35% endosulfan) at concentrations more likely to reach the estuaries by the sugar cane crops runoff. A methodological proposal for microcosms was tested for tropical estuaries. The nominal concentrations (0.001, 0.005, 0.01, 0.05, 0.10, 0.20, 0.35 and 0.55 $\mu g.g^{-1}$ + control) and experimental times (0, 1, 4, 8 and 16 days) were adopted. Nominal and measured endosulfan concentrations in Thiodan showed highly significant correlation with losses below 60%. Significant differences were detected for meiofauna structure between experimental days and concentrations tested. The organochlorine Thiodan measured as endosulfan concentrations had affected the Kinorhyncha group on Day 8 and the Simple Linear Regression explained part of densities variation. There were no significant concentration effects on microphytobenthos and its concentration was not related to groups' feeding. Despite the moderate to high volatilization rate of the endosufan observed in the end of the experiment, it is suggested the absence of mortality patterns due to pesticide bioavailability related to both sedimentary factors and the presence of microalgae. Possible replacement of sensitive by tolerant species or predominance of resistant species in each meiofauna group throughout the experiment is another hypothesis to be discussed.

Keywords: meiofauna, microcosm, pesticide, endosulfan, Thiodan, sugar cane, microphytobenthos.

RESUMO

Efeitos do Endosulfan (Thiodan 35 EC®) sobre a Comunidade e Meiofauna Através de uma Abordagem de Microcosmos

Um experimento de microcosmo com a comunidade natural de meiofauna foi realizado para testar os efeitos do pesticida organoclorado Thiodan 35 CE (35% de endosufan) nas concentrações mais prováveis que atingem os estuários através do runoff da cana-de-açúcar. Uma proposta metodológica para microcosmos foi testada em estuários tropicais. A concentrações nominais (0,001, 0,005, 0,01, 0,05, 0,10, 0,20, 0,35 e 0,55 $\mu g.g^{-1}$ + controle) e os tempos experimentais (0, 1, 4, 8 e 16 dias) foram adotados. As concentrações nominais e medidas de endosulfan do Thiodan apresentaram correlação altamente significativa com perdas inferiores a 60%. Foram detectadas diferenças significativas para a estrutura da meiofauna entre os dias experimentais e as concentrações testadas. As concentrações do organoclorado medidas como endolsulfan presentes no Thiodan afetaram o grupo Kinorhyncha no dia 8 e a regressão linear simples explicou parte da variação da densidade. Não houve efeito significativo na concentração de microfitobentos e sua concentração não esteve relacionada à alimentação dos grupos. Apesar da taxa de volatilização de moderada a alta do endosufan observado ao final do experimento, sugere-se que a ausência de padrões de mortalidade deva-se a biodisponibilidade dos pesticidas relacionada tanto a fatores sedimentares como a presença de microalgas. Uma possível substituição de espécies sensíveis por espécies tolerantes ou a predominância de espécies resistentes em cada grupo meiofauna ao longo do experimento é outra hipótese a ser discutida.

Palavras-chave: meiofauna, microcosmo, pesticidas, endosulfan, Thiodan, cana de açúcar, microfitobentos.

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INTRODUCTION

In estuarine systems of Pernambuco, the development of industrial and agricultural activities constitutes the main source of contamination in these areas, as a consequence of unplanned human occupation and mangrove degradation (CPRH, 2006). In this sense, sugar cane cultivation should be highlighted in Pernambuco: the State is the 5th national producer in the category (IBGE, 2005) and this activity covers about 40% of the north coast (CPRH, 2003). The major problem related to this practice derives from the management of pests in crops, such as termites (Isoptera), which is primarily performed with the application of long residual power insecticides (Fontes, 1995). Organochlorine pesticides are known for their environmental persistence, thus due to its high toxicity and bioaccumulation in organisms, these compounds offer a great risk to ecosystems. Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo(e) dioxathiepin-3-oxide, CAS No. 115-29-7) is a broad-spectrum chlorinated cyclodiene. Its technical preparation consists of two stereo isomers, α- and β-endosulfan in an approximate ratio of 7:3 (Awasthi *et al*., 2003). The half-life for degradation of α-endosulfan is about one to three months whereas that of β-endosulfan and endosulfan sulfate can be from two to six years depending on environmental conditions (Wan *et al*., 2005). One of the insecticides used in sugar cane crops is the Thiodan 35 EC®, an emulsifiable concentrate containing 35% endosulfan in the formulation. This compound was licensed by the Ministry of Agriculture under the No. 1048793 as an insecticide-acaricide with good stability, long persistence and residual power of 50 days; due to such properties, it is classified as highly toxic (class II) and environmental class I – highly dangerous to the environment (Bayer CropScience, 2006). However, after the Public Consultation No. 60 published by the Agência Nacional de Vigilância Sanitária (ANVISA) through the "Diário Oficial da União" on September 4th, 2009 which lasted two months, the final position regarding banning (or not) the active ingredient endosulfan in Brazil has yet been taken and depends on the joint decision of three agencies of pesticides registering in the country: ANVISA, IBAMA (Brazilian Institute of Environment and Natural Resources) and MAPA (Ministry of Agriculture, Livestock and Supply).

In estuarine sediments meiofauna are excellent organisms for studying pollution once these animals facilitate biomineralization of organic material, serve as food for several higher trophic levels and show high sensitivity to anthropogenic inputs. In addition, their intimate association and dependence on sedimentary environments plus high abundance and benthic larvae make them a good tool to assess effects of contaminants (see Coull & Chandler, (1992) for review). For meiobenthos, cause and effect relations at the community level can be established through controlled manipulation of environmental variables in microcosms. Given the extremely adverse environmental conditions in estuaries, it is expected that the meiofauna is able to withstand laboratory manipulation (Austen & McEvoy, 1997a). Due to most meiofauna produce multiple generations per year, community changes can be measured over the time scale at which such experiments can be realistically maintained (Austen, 1989).

Therefore, the aim of this study was to evaluate the effects of Thiodan measured as endosulfan concentrations that may reach the estuaries of Pernambuco by runoff of the pesticide used in sugar cane crops on the meiofauna community through a microcosm approach.

METHODS

Sampling site

The sampling site chosen for collection of the sediment that composed the microcosm experiment is a mudflat in the Canal de Santa Cruz, part of the estuarine complex of Itamaracá Island (7º 46' 24.55" S and 34º 52' 59.95" W) on the north coast of Pernambuco State (Brazil), about 50 km from the capital, Recife. According to Santos *et al.* (2009), this site presents strongly reduced sediment conditions with Eh (redox potential) values always more negative than -100 mV at 5 cm depth in addition to values above 3% for organic matter and 25% for silt and clay.

Laboratory and field procedures

Contamination of stock sediment with endosulfan (Thiodan 35 EC)

A sediment sample (500 g) was collected at the sampling site. It was defaunated by freeze-thawing and fortified with 0.5 mL of Thiodan 35 EC® (35% m/v Endosulfan as active ingredient + 65% inert compounds). Three replicates (100 g) were sent for analysis of the endosulfan concentration adsorbed to the sediment by gas chromatography (GC-ECD) following the CETESB protocols described in Casarini *et al.* (2001). The remaining sediment was frozen and used in microcosm experiments. From the endosulfan concentrations obtained in those three replicates analyzed, the average endosulfan concentration present in Thiodan in stock sediment was calculated as 12 + 4.8 $\mu g.g^{-1}$ (average + standard error) and this value was the starting point to obtain the target nominal concentrations in the experiment. Inert compounds were not included on the toxicity investigation.

Experimental design

Most laboratory studies with animals of meiofauna were developed in bioassays with organisms previously isolated and cultivated using Petri dishes, or with natural communities through the direct use of fresh sediment collected in field and maintained in microcosm environments (see Coull & Chandler, (1992) for review plus Austen & McEvoy, 1997a, b), there being no rigor/concern in maintaining the vertical stratification of the sediment. This study followed the methodology applied in Austen & Warwick (1995) with modifications, which made

direct use of the cores collected in the field as part of the microcosm environment. The study performed by Austen & Warwick (1995) was a mesocosm approach using sediment of two estuaries from temperate region with five treatments in two experimental times (initial and final) maintained during 16 weeks under temperatures ranging between 15 and 20 °C and making use of corers with 6.4 cm in diameter grouped in tanks of 1 m^3. The idea of using syringes (cores) in a microcosm experiment has primarily considered the difficult in successive manual samplings in the microenvironment without disturbing the sediment and consequently the community, as well as the natural strongly reducing conditions in estuarine sediments which are stressed in bioassays maintained under the temperature of a tropical region. Thus, syringes used in this experiment were drilled at 1 cm from the base and had their plungers also drilled in the center and wrapped with a mesh of 32 mm aperture in order to maintain water circulation and aeration of the sediment in the lower portion of each syringe and its vertical stratification preserved. (Figure 1a). Therefore, "miniaturization" of the experimental scale from meso- to microcosm was a significant change in the present study which enabled the development of a study with many treatments and various experimental times. In addition, all adjustments performed in the syringes allowed the experiment to be successful regarding the high experimental temperature (24 °C) required to simulate a tropical estuarine environment.

From the amount of active ingredient (2100 $g.ha^{-1}$) recommended to be used in sugar cane crops (Bayer CropScience, 2006), considering its runoff concentrations observed in various studies for water and sediments (*e.g.* Kennedy *et al*., 2001) and the results obtained in the works of Chandler & Scott (1991), Barry & Logan (1998) and Scott *et al*. (2002), the following nominal endosulfan concentrations present in the Thiodan were chosen for the microcosm experiment: 0.001, 0.005, 0.01, 0.05, 0.10, 0.20, 0.35, 0.55 $\mu g.g^{-1}$. The experimental design of 5 independent replicates for each microcosm treatment and control was designed. The total experimental period of 16 days was chosen taking into account the meiofauna's short life cycle – since many meiofaunal groups undergo complete generations in 2–4 weeks (Coull, 1999); the endosulfan chemical properties and the choice for an experiment with no interference with food supply from beginning to end. The following experimental times were used for samplings in the laboratory: Day 0 before endolsufan (Thiodan 35 EC) addition and Days 1, 4, 8 and 16 before endolsufan (Thiodan 35 EC) addition. Days 1 and 4 corresponded to 24 and 96 h, initial and final experimental times commonly used in experiments of acute contaminants effects, while days 8 and 16 would be sufficient time to evaluate chronic effects considering meiofauna's life cycle as well as to observe patterns of decline or recovery of these animals.

Field sampling for the microcosm composition

The sediment for microcosm composition was collected in a stretch of approximately 5 m in length in a muddy flat of Canal de Santa Cruz – low tide (0.4 m) – in March 2009. A sum of 495 plastic corers (syringes) with different volumes to a depth of 3 cm was used in the samplings. The samples for meiofauna extraction were collected with 225 syringes of 2.01 cm^2 (10 mL); those designed to examine microphytobenthic concentrations were collected with 225 syringes of 1.13 cm^2 (5 mL) and the sediment for analysis of final endosulfan concentrations with 45 syringes of 7.06 cm^2 (60 mL). Each syringe containing sediment was internally sealed by the lower opening with its own plunger at the sampling time, fixed in a Styrofoam plate, stored in covered plastic boxes (to prevent desiccation, contamination and preserve the sediment oxygen) and transported to the laboratory for experiment assembly (1h- after sampling).

Microcosm experiment

In the laboratory, syringes containing sediment were aleatory grouped to form each microcosm as follows: 5 syringes of 10 mL (meiofauna), 5 syringes of 5 mL (microphytobenthos) and 1 syringe of 60 mL (final endosulfan concentration as Thiodan 35 EC). Each set of syringes was tied with a rubber band, conditioned in 1 L beaker and filled it with 600 mL of filtered water of the estuary. Microcosms consisted therefore of 1 L glass beakers aerated with air diffusers and covered with plastic film functioning as micro-aquarium – each beaker representing a replicate in the experiment – (Figure 1b). A total of 45 beakers composed the microcosms (5 replicates × (8 treatments + 1 control)). The microcosms were maintained in 14:10 h L:D photoperiod to a constant experimental temperature of 24 + 2 °C (to simulate the natural environment) and salinity corrected to 35 with distilled water when necessary. The complete experiment assembly has taken 12 hours; therefore, there was one day for animals' acclimatization to the experimental environment before the start counting on Day 0.

The first sampling was performed on Day 0 before endolsufan (Thiodan 35 EC) addition removing one 10 mL-syringe (meiofauna) and one 5 mL-syringe (microphytobenthos). After 12 hours, microscosm were contaminated: 5 beakers of each treatment were contaminated with the addition of a calculated volume of stock sediment to obtain the 8 target concentrations. Subsequent samplings occurred on 1, 4, 8 and 16 experimental days for meiofauna and microphytobenthos; and only on the last day for the final endosulfan (Thiodan 35 EC) concentrations.

Meiofauna

Meiofauna samples were fixed in 4% formaldehyde and stained with Rose Bengal after sampling in each experimental day. Samples processing followed the methods described in Giere (2009). Densities were expressed in ind.10 cm^{-2}.

Microphytobenthos

Sediment samples for microphytobenthos were frozen until extraction. The extraction of chlorophyll-*a* and phaeopigments followed the method of Colijn & Dijkema (1981) with modifications and the calculations were performed by the equations of Lorenzen (1967). The 750 nm wavelength was used to eliminate the interference of turbidity in the sample. Results were expressed in $\mu g.cm^{-2}$.

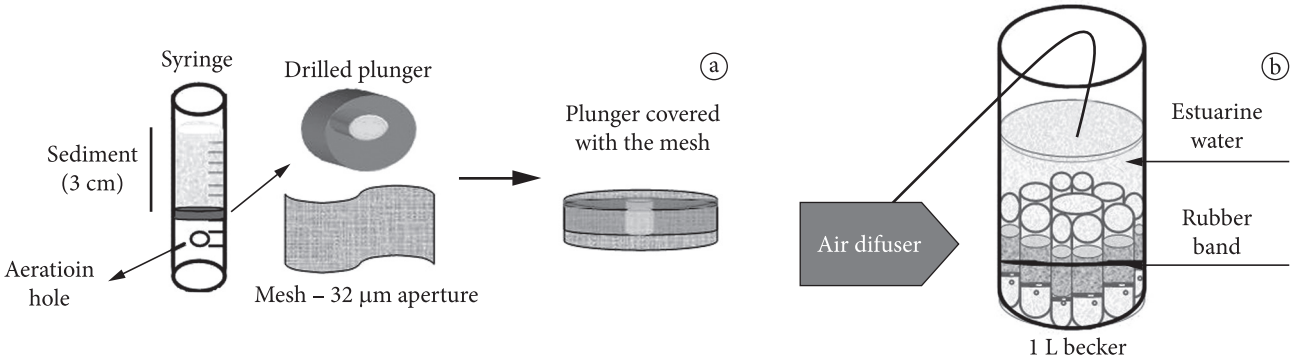

Figure 1. Scheme illustrating the design of syringes used in the microcosm experiment (a) and the assembly of syringes in the becker representing the microcosm environment (b).

Statistical analysis

The multivariate statistical analysis followed the methods described by Clarke & Warwick (1994) and Clarke (1993). The analyses were performed using the softwares Primer 5.2.4 and BioEstat 3.0. The Analysis of Variance (1-factor ANOVA) followed by *post hoc* Tukey's and the Analysis of Similarity with one factor (one-way crossed ANOSIM) were used to test the system's homogeneity regarding microphytobenthos and meiofauna structure on Day 0, respectively. The Analysis of Similarity with two factors (two-way crossed ANOSIM) was used to test possible differences on meiofauna structure between experimental days and the different treatments. Similarity matrices for ANOSIM analyses were calculated from the meiofauna density data using the Bray-Curtis measure and fourth-root transformation in order to reduce asymmetry and stabilize the variance of samples. Analysis of non-metric Multi-Dimensional Scaling (nMDS) from the meiofauna density data was used for plotting the meiofauna community structure tested in the ANOSIM analyses. Simple Linear Regression Analysis was used to verify the relationship between initial and final concentrations of total endosulfan present in the Thiodan. In addition, it was applied to meiofauna groups, total meiofauna and microphytobenthos with endosulfan concentrations as independent variable to investigate Thiodan influence on the fauna and microflora throughout the experimental days. Simple Linear Regression analysis was also used to test possible influences of chlorophyll-*a* and phaeopigments on meiofauna groups and total meiofauna. Data were checked for conformity with the assumptions required for valid parametric analysis and those variables which showed significant heteroscedasticity were 4th root-transformed. For all analysis $p < 0.05$ was adopted as significance level and the Bonferroni correction was applied to regression analyses with $p < 0.01$ being considered as significance level.

RESULTS

**** Nominal vs final endosulfan concentrations: validation and application of results***

The chemical analyses on final concentrations of α, β and Total-endosulfan present in the Thiodan in each treatment showed decrease in relation to the nominal concentrations. In the sediment only in treatments of highest nominal concentrations (0.20, 0.35 and 0.55 µg.g^{-1}) was indeed detected endosulfan. There was an average loss of 59% for α-endosulfan, 9.5% for β-endosulfan and 44% for total endosulfan in 16 days. In addition, the average ratio between α-Endosulfan and β-Endosulfan was 0.97 in the end of experiment. Figure 2 shows the linear tendency line considering total endosulfan concentrations (nominal and final) present in the Thiodan. According to the Simple Linear Regression Analysis there is a highly significant relationship ($R^2 = 0.9248$; $F = 99.3860$; $p = 0.0001$) between nominal and final concentration of total endosulfan, however, final measures were shown to be inaccurate in lower concentrations in relation to the nominal concentrations applied.

Validation of the experimental set on day 0: the issue of variability (heterogeneity) among replicates:

Microcosm's replicate data set (Replicates A, B, C, D and E of each Treatment) was evaluated on Day 0 (prior to contamination with endosulfan as Thiodan 35 EC) in order to validate the system as a set without significant heterogeneity among replicates for meiofauna and microphytobenthic communities. According to the nMDS plot and taking into account that no single R statistic from the 1-way ANOSIM was superior to 0.25, there was no significant heterogeneity among replicates for meiofauna community in the beginning of the experiment (Figure 3a). The same pattern was shown by the 1-way ANOVA for chlorophyll-*a* ($F(4;40) = 0.481$; $p > 0.05$) and phaeopigments concentrations ($F(4;40) = 0.595$; $p > 0.05$). However, due to oxygenation problems in the system for about three hours on Day 1, the replicates C and D were excluded from all analyses.

Endosufan effects on meiofauna structure

According to the 2-way ANOSIM (Days × Concentrations), meiofauna community structure presented significant differences for both factors tested (Table 1). For the experimental days, differences were observed between: D0 × D8, D0 × D16, D1 × D8, D1 × D16 and D4 × D16. Regarding to treatments, there were differences between 0 × 0.001 µg.g^{-1}, 0.001 × 0.01 µg.g^{-1}, 0.001 × 0.05 µg.g^{-1} (Figure 3b and 3c).

Nematodes were dominant along the experiment. Moreover, there was constancy throughout the experiment and concentrations in relation to meiofauna composition: Nematoda, Copepoda, Nauplii, Ostracoda and Kinorhyncha. No clear dose-response pattern was observed regarding to densities for any group or total meiofauna in relation to the different concentration levels analyzed. According to the in Simple Linear Regression Analysis, only Kinorhyncha densities had 29% of its variation positively explained by concentrations applied on Day 8 ($R^2 = 0.2960$; $F = 11.9299$; $p = 0.002$). When each concentration is observed in each group and the total meiofauna throughout the experiment, there is a trend in reducing densities of all groups and total meiofauna, though not significantly, highlighting the group Copepoda for which this trend was clearly observed in all concentrations during the experiment (Figure 4).

Microphytobenthos: contribution or interference in the data interpretation?

Microphytobenthos was evaluated with regard to Thiodan 35 EC (which contains about 35% of endosulfan) effects throughout the experiment. In addition, the influence of chlorophyll-a on meiofauna groups and total meiofauna was also investigated. According to the Simple Regression

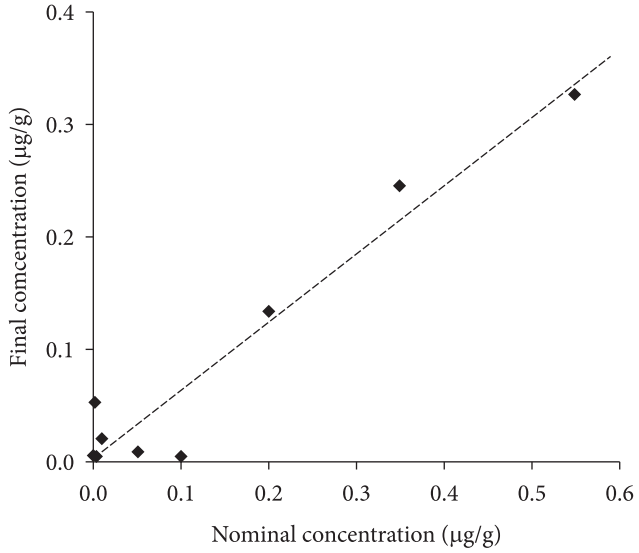

Figure 2. Simple Linear Regression Analysis between initial and final concentrations of total endosulfan present in the Thiodan pesticide.

Table 1. Differences on meiofauna structure between Days and Concentrations according to the 2-way ANOSIM pairwise tests ($p < 0.05$).

2-way ANOSIM – pairwise tests			
Experimental days	**R statistic**	**p (%)**	**Permutations**
D0 × D8	0.667	0.01	10,000
D0 × D16	0.737	0.01	10,000
D1 × D8	0.539	0.01	10,000
D1 × D16	0.683	0.01	10,000
D4 × D16	0.621	0.01	10,000
Concentrations (ug.g^{-1})	**R statistic**	**p (%)**	**Permutations**
0 × 0.001	0.593	0.02	10,000
0.001 × 0.01	0.504	0.05	10,000
0.001 × 0.55	0.533	0.02	10,000

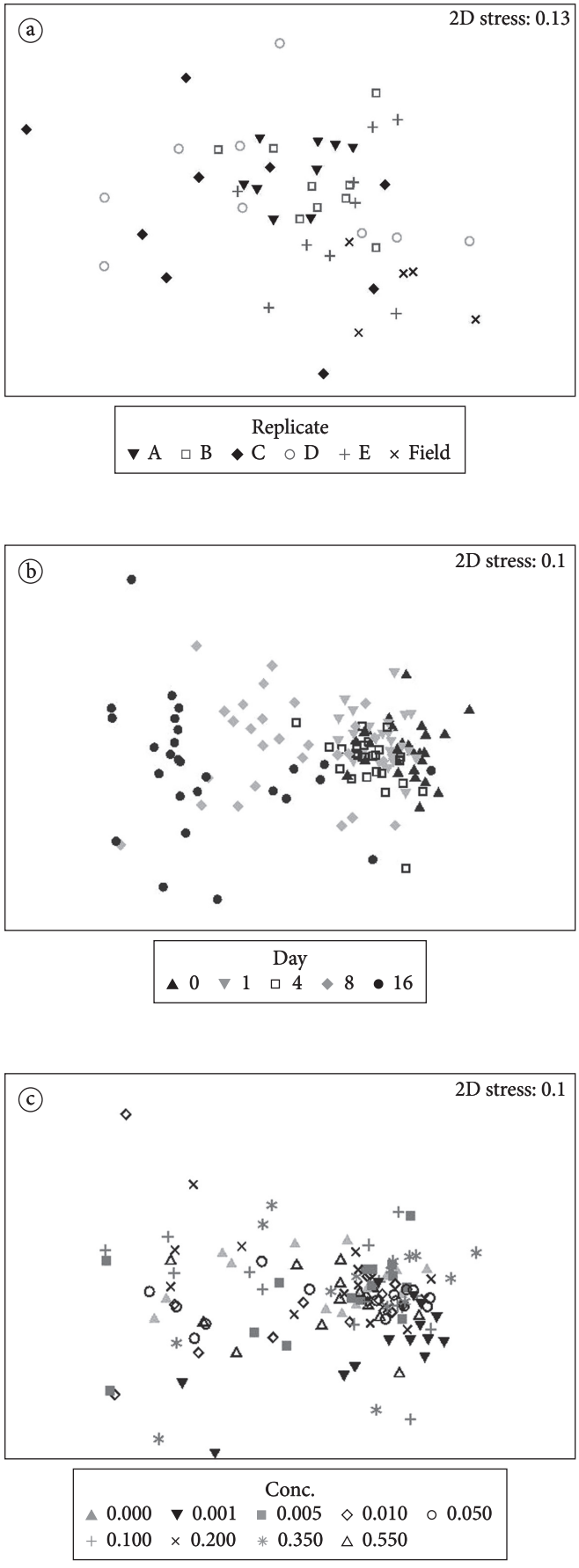

Figure 3. MDS plot of meiofauna structure among all replicates on Day 0 (a) and throughout experimental days (b) and concentrations (c) applied.

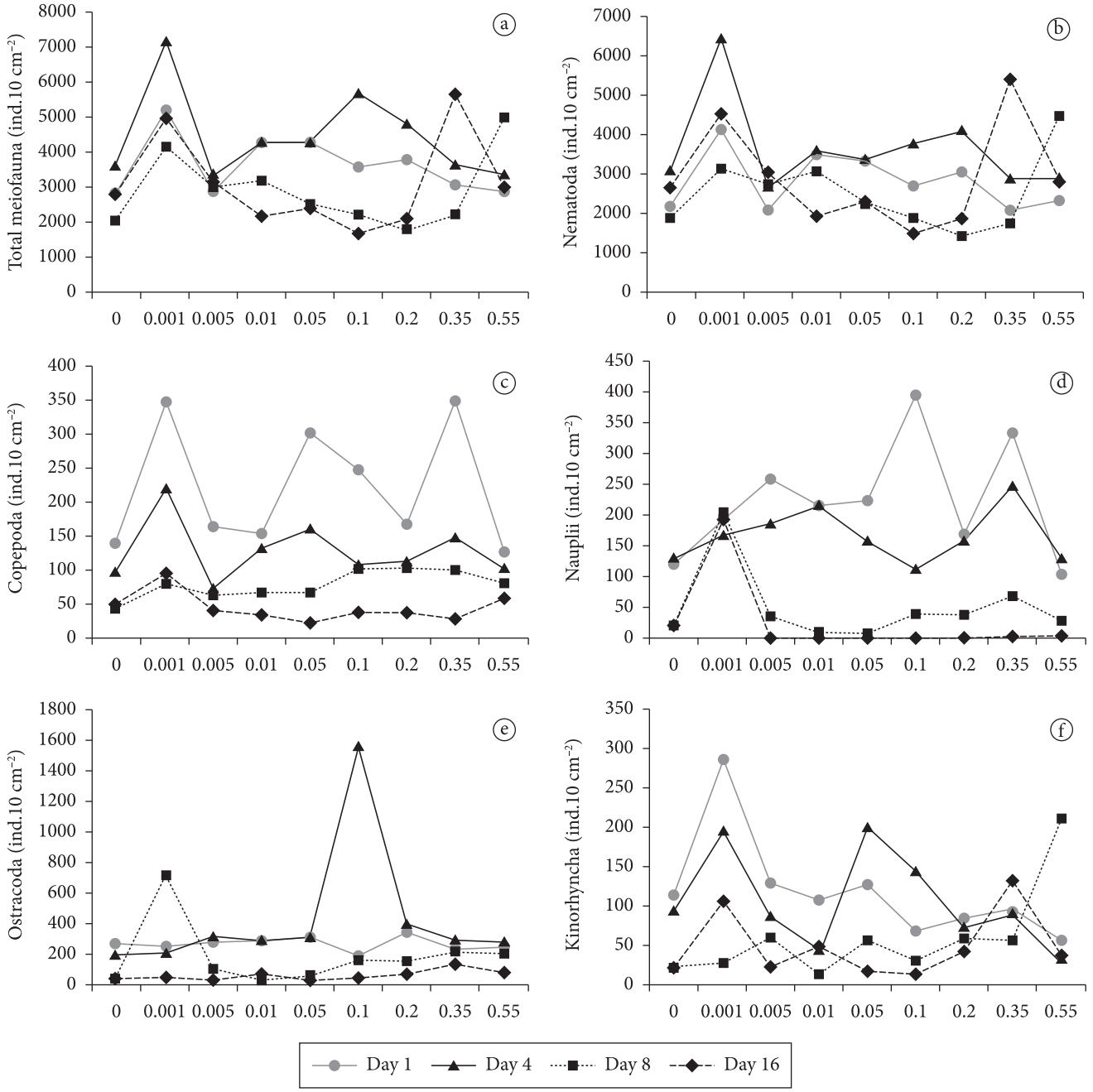

Figure 4. Average densities of Total Meiofauna (a), Nematoda (b), Copepoda (c), Nauplii (d), Ostracoda (e) and Kinorhyncha (f) throughout experimental days and concentrations applied.

Analysis, the treatments applied were not responsible for variations on microphytobenthic concentrations ($p > 0.05$) as well as microalgae have not interfered significantly on meiofaunal densities.

DISCUSSION

Nominal vs final endosulfan concentrations: validation and application of results

Endosulfan is equivalent to two insecticides with different water solubility and volatility since it is composed of two isomers. The α-endosulfan is more volatile (vp = 0.006 mm Hg) and less water soluble (2.29 $mg.L^{-1}$) compared to β-endosulfan (vp = 0.003 mm Hg and water solubility = 31.1 $mg.L^{-1}$) (Antonious & Byers, 1997). In addition to these properties, other factors such as different photodecomposition, alkaline hydrolysis, biotic metabolism can interfere in the persistence both isomers. Also, aerobic conditions can promote endosulfan biotransformation, being the endosulfan sulfate its major metabolite formed (Awasthi *et al*., 2003), despite the various forms of loss and/or transformation of endosulfan, the volatilization is reported as the main route of loss in aerated bioassays with none or incomplete sealing of containers (Guerin & Kennedy, 1992). This hypothesis is the most reasonable and likely to explain the losses observed in this study though some

of the endosulfan applied through the Thiodan pesticide may have been degraded to endosulfan sulfate (unmeasured values). Both endosulfan isomers exhibited relatively short half-lives and the present results are consistent with other microcosm studies that reported significant losses of more than 50% in two weeks or even during the first days after the application of initial nominal concentrations (Guerin & Kennedy, 1992; Berrill *et al*. 1998; DeLorenzo *et al*. 1999). In addition, according to Laabs *et al*. (2007), small microscosm systems (< 1 L) have a low surface/volume ratio that favors endosulfan hydrolysis and losses by edge effects, fact also observed previously in the studies of Flemer *et al*. (1995) and Barry & Logan (1998).

Even losses by volatilization been high; the final concentrations did not compromise the goal of working within the range of probable concentrations in runoff events. However, due to the method's measurement accuracy (0.001 g/g detection limit), the fit between observed and expected was not good in the lower concentrations. According to Kennedy *et al*. (2001), in normal conditions, irrigation waters account for 1 to 1.5% of total endosulfan transported off-field in one year but the total endosulfan removed from a field by storms may transport up to 10% of the endosulfan on-field at the time of the event, particularly if it occurs soon after endosulfan application. Thus, considering that endosulfan application through the Thiodan in the sugar cane crops of Pernambuco is the recommended 2100 $g.ha^{-1}$, this implies in 21 $\mu g.cm^{-2}$ applications. Extrapolating this value to each microcosm environment (Area = 78 cm^2 and $Sediment_{(w)}$ = 383 g), the value applied in field corresponds to 4.2 $\mu g.g^{-1}$ but only 1.5% of this value (0.06 $\mu g.g^{-1}$) is expected as runoff to the estuary under normal conditions. Therefore, the highest final concentration of endosulfan present in the Thiodan in this study was well above values expected under normal conditions and similar to those that may be expected under extreme events.

Experimental validation of samples set on day 0: the issue of variability (heterogeneity) among replicates:

Since the aim of ecotoxicology is to measure and predict chemical effects on the natural environment, there is another issue related to the microcosm approach itself: the inherent variability of multi-species biological systems and the complexity of resulting data (Barry & Logan, 1998). This problem was considered and evaluated through the analysis of the microcosm system on Day 0. The microcosm system was validated as sufficiently homogeneous on Day 0 taking into account the considerations of Clarke & Warwick (1994) on the R statistic value and the nMDS stress value. According to the authors, the R statistic itself is an useful comparative measure of the degree of separation of sites or treatments and values much lower than 0.5 even with significant *p* values, indicate no significant differences, also nMDS stress values < 0.2 still give potentially useful 2-dimensional pictures which does happened in this work. In addition, the ANOVA results for the whole replicate set of microphytobenthos reinforce those obtained by similarity analysis performed for meiofauna community structure. The considerations of Clarke & Warwick (1994) were not only crucial in the initial validation of the experiment, but also were followed up for all the multivariate analysis applied throughout this study.

Endosulfan effects on meiofauna structure

Microcosms based on natural communities often provide a closer approximation to natural ecosystems, since their composition, structure and function may approximate the organization of larger systems and thus indicate that interactions observed in the laboratory should reflect at least a subset of biotic and chemical processes occurring in the environment (Barry & Logan., 1998). The constancy in terms of meiofauna groups throughout the experiment reflects the natural environment and is also similar to those observed in other microcosm studies (*e.g.* Flemer *et al*., 1995). The absence of patterns in response to the exposed contamination shows that pesticide concentrations that may reach estuaries of Pernambuco by the runoff of Thiodan have not negatively affected the local meiofauna. In this sense, data from literature indicates two thematic possibilities: the issue of bioavailability and species-specific responses. Bioavailability is affected by numerous external variables such as organic carbon, pH, redox state and salinity, which control the speciation of a toxicant and the concentration of the active ingredient available for uptake (Meador *et al*., 1993). After toxicants enter marine organisms via food, water or sediment, they can be stored or eliminated. The kinetics of both storage and elimination are affected by the organism's metabolism and even among closely related groups, occupying similar habitats, there might be marked variations in toxicants bioaccumulation (Schratzberger *et al*., 2002). In addition, toxicants use to be less toxic in sediments than in water conditions (Austen & McEvoy, 1997b). In the "sediment situation", many toxicants may not be bioavailable to meiofauna (Coull & Chandler, 1992) due to their toxicities may be reduced by several factors such as chelation, complexation, or by binding to organic ligands and colloidal aggregations on the sediment surface (Giere, 2009). On one hand, Scott *et al*. (1999) determined a LC50 of 1.01 $\mu g.L^{-1}$ for the estuarine grass shrimp *Paleomonetes pugio* by field and laboratory bioassays due to gravid female grass shrimp populations have elevated levels of P-glycoprotein (P-gp), a multidrug resistance protein, which may transport various pesticides across cellular membranes. On the other hand, Leonard *et al*. (2001) determined a 10-d no observed effect using 42 $\mu g.kg^{-1}$ sediment-associated endosulfan for the epibenthic mayfly *Jappa kutera.* These results suggesting that pulse exposures of endosulfan in the water column following storm runoff may be more acutely toxic to riverine biota than in contaminated bottom sediment. Therefore, it is the bioavailability and not the absolute concentration of pollutants measured in water or sediment that determines the noxious effects on the benthic environment (Giere, 2009). The concentration levels evaluated in this study ranged between 0.004 to 0.32 $\mu g.g^{-1}$ measured at the end of experiment or between 0.001 to 0.55 $\mu g.g^{-1}$ nominal at the experiment start and no significant mortality for total meiofauna or groups individually was observed. Although kinorhynchs present their bodies covered by a strongly water-repelling cuticle (Giere, 2009), a fact that favors the passive uptake of pesticides, this group showed

tolerant/resistant to the endosulfan, which provided increased densities in this group on the Day 8 of the experiment. However, since most studies on toxicity were and still are performed with Copepoda and Nematoda or the meiofauna as a whole, there are no studies on pesticide effects on Kinorhyncha for further comparisons. Schratzberger *et al*. (2002) evaluating the TBT effects on nematode species point out that diet is indeed known to be a mechanism capable of influencing body burden and this may be more significant than uptake from water; it clearly makes sense in this study since nematodes (which have a permeable cuticle) were not significantly affected by endosulfan concentrations tested. Wan *et al*. (2005) determined LC50 for endosulfan (α + β) for *Daphnia magna* (840 $\mu g.L^{-1}$), the salmonid fish *Oncorhynchus mykiss* (0.7 $\mu g.L^{-1}$) and the amphipod *Hyalella azteca* (5.7 $\mu g.L^{-1}$) in simulated field water, suggesting that the combination of these compounds together with their oxidized sulfate metabolite is most potent to both crustaceans and fish (at least as indicated in short-term studies) and that in such combination, isomers synergize one another to enhance biological activity. Chandler & Scott (1991), while conducting sediment-bound bioassays, found that survival of the benthic copepod *Nannopus palus*tris was only affected by 19% at concentration as high as 200 $\mu g.kg^{-1}$ and the same concentration caused no effect in the copepod *Pseudobradya pulchella*. However, the same concentration (together with other contaminants) caused mortality in harpacticoids of the genus *Mesochra* and cyplopoids of the genus *Halicyclops*, in addition to decrease in abundance and number of taxa for meiofauna community (Bollmohr *et al.*, 2009). Specifically with regard to Copepoda Harpacticoida in this study, the muddy flat in the Canal de Santa Cruz is well studied. The genera *Robertsonia, Cletocamptus* and *Nannopus* are constantly present and dominant in the sediment of this site (Santos *et al*., 2000.) in addition to be genera with species commonly resistant to anthropogenic stresses. In contrast, the genus *Halectinosoma,* also constantly present (Santos *et al*., 2000) is usually much more sensitive to the various types of disturbance (Souza-Santos *et al*., 2004). According to Bejarano & Chandler (2003), species react differently to the same pollutant. Therefore, accepting the species-specific differences in sensitivities to aqueous pollution that have been reported among most meiobenthic taxa (Coull & Chandler, 1992), it is likely that most sensitive species have been replaced by other more tolerant throughout the experiment or even that the Harpacticoida group was composed in most by resistant genus/species since the beginning, which may explain the absence of patterns in this study. In addition, sometimes shortly after a pollution event, the overall abundance of meiofauna may increase (depending on the species, the nature and concentration of the pollutant, etc.) superficially suggesting a negligible impact and leading to wrong conclusions about the absence of effects (Giere, 2009).

Microphytobenthos: contribution or interference in the data interpretation?

Microphytobenthos is not only of considerable nutritional importance to meiofauna; it also alters the structure and chemistry of the sediment (Giere, 2009). Results of DeLorenzo *et al*. (1999) suggest that endosulfan primarily reduces bacterial abundance, the number of cyanobacteria and phototrophic biomass; however, this effect was not observed in this study. This fact is probably due to the concentrations used, since these authors found no effects on chlorophyll-*a* using endosulfan concentrations as high as 10 $mg.L^{-1}$. The regression analysis indicated that chlorophyll-*a* had no effect on the densities of meiofaunal groups. It was an unexpected result since not only many harpacticoids (Montagna *et al*., 1989) but also ostracods (Giere, 2009) and kinorhynchs in fine/estuarine sediments (Neuhaus & Higgins, 2002) typically use to feed on diatoms and be influenced by microalgae (Cibic *et al*., 2009). However, to what extent can this be considered as an indirect effect of endosulfan? Whether on the one hand endosulfan concentrations were not high enough to compromise the algal growth; on the other hand it must be remembered that the algae are particularly likely to take up pesticides because they generally have a high surface area to volume ratio (DeLorenzo *et al*., 2002). According to Barry & Logan (1998), endosulfan can undergo reversible binding dynamics with the plant material and when large amounts of phytoplankton are present in the water, relatively little of the pesticide may be bioavailable to organisms. The high chlorophyll-a concentrations observed in this study reinforce the basic idea of increased or reduced toxicity to animals which feed or not directly on microalgae, thus being useful to associate this biological component is studies involving meiofauna responses to several contaminants.

FINAL REMARKS

Endosulfan concentrations (together with other compounds present in the commercial Thiodan 35 EC pesticide) present in the Thiodan that supposedly hit the Pernambuco estuaries by the pesticide runoff seem not affecting meiofauna groups negatively; though this might be possible focusing on species level. The different threshold of tolerance reported for the three genera/species of Copepoda Harpacticoida for the same endosulfan concentrations in sediment is a good example of how variable the biological response may be and it is a subject that deserves special attention. As discussed above, sedimentary and biological factors, which also occur in the environment, hinder the evaluation proposed in the beginning of the study. However, environmental issues of extreme importance as the problem here addressed for the estuaries of the North Coast of Pernambuco cannot be neglected or taken with less concern, especially considering that sugar cane cultivation covers the South-Central and North-Northeast portion in Brazilian coast. The present results point the great complexity of biological systems and the inherent difficulty of separating causes and effects that frequently act synergistically with the environmental variables. This work provides an important contribution to the information gap involving meiofauna and the pesticides universe; in addition this work provides new information about possible resistant responses of the Kinorhyncha group to pesticide effects. The methodological proposal used in this study is the first microcosm approach performed in Brazil making direct use of corers inside the microenvironment as part of the experiment. This approach allowed avoiding problems related to the sediment structure and its oxygenation that certainly would be reflected in meiofauna community and would compromise the integrity of the results. In addition, the microcosm approach tested in the

present study can be reproduced for any estuarine community or even other environments, as well as its applicability can be extended to any type of pollutant. Finally, in addition to an important methodological advance, this study adds knowledge to the background of microcosm approaches with natural communities that due to several logistical adversities are still a largely unexplored field in developing countries.

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