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Carbofuran effects in soil nematode communities: Using trait and taxonomic based approaches

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ABSTRACT

This work intends to implement the use of native soil nematode communities in ecotoxicological tests using a model pesticide and two geographically nematode communities (Mediterranean and subtropical) in order to obtain new perspectives on the evaluation of the toxic potential of chemical substances.

The environmental condition of the nematode communities was described using a trait-based approach (grouping the organisms according to their feeding traits) and a traditional taxonomic method (identification to family level). Effects on total nematode abundance, number of families and abundance of nematode feeding groups as well as potential shifts in both trophic and family structure were assessed.

Agricultural soils from Curitiba (Brazil) and Coimbra (Portugal) were sampled and the corresponding nematode communities were extracted. Part of the collected soil was defaunated and spiked with four doses of a carbofuran commercial formulation. Afterwards each of the replicates was inoculated with a nematode suspension containing ≈200 or 300 nematodes. After 14 and 28 d of exposure the nematodes were extracted, counted and identified at family level and separately classified according to their feeding traits. The patterns of nematode responses revealed a decrease in the total abundance and a reduction in the number of families. Despite the similar effects observed for both communities, statistically significant toxic effects were only found within the Portuguese community. The total nematode abundance was significantly reduced at the highest carbofuran concentrations and significant shifts in the family structure were detected. However, the trophic structure, i.e., the contribution of each feeding group for the overall community structure, did not significantly change along the contamination gradient. Results showed that using such a trait-based approach may increase the ecological relevance of toxicity data, by establishing communalities in the response to a chemical from two different taxonomic communities, although with potential loss of information on biodiversity of the communities.

1. Introduction

Ecological Risk Assessment (ERA) based on ecological/functional traits (TERA—Trait-Based Risk Assessment; Baird et al., 2008) advocates the use of morphological/physiological/ecological characteristics of organisms to describe the effects of toxic substances or other stress factors at the community level, in terms of species abundance, diversity, distribution and interactions with other species and the environment (Baird et al., 2008; Clements and Rohr, 2009; Van den Brink, 2008). Scientists working in ERA and/or biomonitoring realized that the description of community responses to stress based only in taxonomic data was a limited approach (Baird et al., 2011). Hence, a good way to enhance a more complete characterization of structure and function of ecosystems would be also to express the status and/or responses of the communities as a combination of traits (Baird et al., 2011; De Bello et al., 2010; Vandewalle et al., 2010).

For the soil system, the use of nematodes, a sensitive group to chemical contamination, can increase the accuracy of predictions

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on the community responses to several forms of pollution induced threats (Bongers and Bongers, 1998; Sochová et al., 2006; Yeates and Bongers, 1999). Indeed, nematodes are ubiquitous in all environments and play a crucial role in the soil system as they intervene in many soil processes and interact with other soil organisms by feeding on them or being their food (Bongers and Ferris, 1999). Moreover, the structure of their mouthparts and pharynx is closely related with their feeding habits and a grouping system based on this particular biological trait – the feeding habit – has been developed (please see the synthesis by Yeates et al., 1993 and references therein). Since they live in the soil interstitial water and have a permeable cuticle, they can be exposed (and adversely affected) to dissolved chemicals (Bongers, 1999; Sochová et al., 2006).

The use of nematodes as test-organisms in soil ecotoxicology has significantly increased over the past two decades. Some laboratory single species tests, assessing the effects of chemicals on nematode reproduction, growth and/or lethality in soil have been proposed and two of them have been standardized (ASTM, 2008; Donkin and Dusenbery, 1993; ISO, 2009; Kammenga et al., 1996; Van Kessel, 1989). Laboratory studies on the effects of pollutants on soil nematodes have been mainly focused on heavy metal toxicity in temperate systems (for a review see Sochová et al., 2006), using a reduced number of nematode species (mostly bacterivorous species like *Caenorhabditis elegans*—Rhabditidae and *Plectus acuminatus*—Plectidae) (e.g. Höss et al., 2002; Kammenga et al., 1996; Sochová et al., 2006). On the other hand, in field (or semi-field, i.e., microcosms) studies, the effects of chemicals on nematode communities are described in terms of changes in feeding and/or life strategies and also by using classical structural endpoints like abundance and diversity (Sochová et al., 2006). However, in such experiments, researchers have to struggle with the high spatial and temporal variability and with the influence of several abiotic parameters affecting the responses of organisms (e.g. Bongers, 1990; Lazarova et al., 2004; Moser et al., 2004).

The present study intends to introduce an innovative approach on testing nematodes in soil ecotoxicology. The effects of soil contamination with a carbofuran commercial formulation were evaluated using a community approach under laboratory conditions. Aiming to improve ecological relevance of the testing strategy, soil native nematode communities, instead of the classical (single) standardized species, were previously extracted from clean soil and exposed to a gradient of contaminated (but defaunated) soil.

Moreover, effects were described using two distinct approaches, comprising functional and structural endpoints. In the trait-based approach, the organisms were grouped according to their feeding traits and changes in the trophic structure (the percentage contribution of each feeding group to the global community structure) and total abundance of each feeding group were assessed. A traditional taxonomic methodology was also used and consisted in the identification of nematodes at family level. Hence, effects on abundance, number of families and family structure were reported.

Our main goal was to contribute to the adoption of the community test approach in soil ecotoxicological testing by comparing the effects of a model pesticide (carbofuran) towards two geographically distinct (Mediterranean and sub-tropical) nematode communities. More specifically, the objectives of the present study were to evaluate and compare the effects of carbofuran applications on the total abundance, total abundance of feeding groups, trophic structure and number of families of two soil nematode communities from different biogeographic regions and, to implement the use of nematode feeding traits as an alternative to taxonomic descriptions when evaluating disturbances at community level.

2. Materials and methods

2.1. Soil sampling and handling

In Brazil, soil samples were collected in Pinhais (Curitiba, Brazil; −25.391667, −49.125000), from an agricultural field with no history of pesticide application or chemical fertilization over the last 6 years. In Portugal, samples were taken from an agricultural soil, located in Carapinheira (Coimbra; 40.209528, −8.657913), with no application of pesticide or chemical fertilizers at least for the last 2 years. A parcel of fallow land was selected at each country ($\approx 15 \times 15$ m in Brazil and $\approx 4 \times 7$ m in Portugal). The vegetation layer was removed and several soil cores, with approximately 10 cm Ø × 10 cm height, were collected in parallel lines. They were mixed, sieved (5 mm) and stored at 4 °C until further processing (two days for the Portuguese soil and one week for Brazilian soil, which was sent to Portugal for testing). Two weeks before the test, part of each soil was defaunated trough two freeze-thawing cycles (48 h freeze at −20 °C and 24 h heating at 65 °C) (Viketoft, 2008).

The soil properties (Table 1) from Portugal were analyzed by DRAPN (Porto, Portugal) as described by Chelinho et al. (2011) and the Brazilian soil by the Department of Soils of UFPR (Curitiba, Brazil) according to Marques and Motta (2003) and EMBRAPA (1997).

2.2. Experimental procedure

As the test with the Brazilian nematode community ran before the Portuguese and in the former a low nematode recovery was detected, it was decided to change some procedures aiming to improve test performance. The incubation period for the microflora and the number of inoculated organisms were increased and the soil moisture level was diminished (see Sections 2.2.1 and 2.2.3).

2.2.1. Soil microflora inoculation

To ensure a rapid recolonization of the soil by the original microflora and to provide food to some groups of nematodes, a soil suspension was prepared with 1000 g of fresh soil and 2000 mL of water, centrifuged twice at 600g during 5 min (Viketoft, 2008). The suspension was passed through a 20 μm sieve, to ensure the absence of nematodes. The inoculation of soil microflora (consisting of Protozoa, Fungi, Bacteria, Actinomycetes and Algae according to Coyne (1999); although not subjected to confirmation by microbiological analysis) was made by mixing 100 mL of soil suspension to 3000 g (Dry Weight, DW) of defaunated soil. This mixture was incubated at 23 ± 1 °C in the dark for 3 or 7 d, respectively, for the Brazilian and Portuguese assays.

2.2.2. Nematode extraction

Fresh soil was used to extract the original nematode community using the tray method (Abrantes et al., 1976) that allows the extraction of large soil samples without the decrease in extraction efficiency of other methods such as Baermann funnel (van Bezooijen, 2006). Based on preliminary assays, several trays were set up ($36 \times 26 \times 5$ cm), each one containing 250 cm^3 of soil (≈ 220 g) spread over paper tissue, supported by a plastic mesh and moist with ≈ 1000 mL of tap water. After 60 h, (22 ± 2 °C), the meshes were removed, briefly drained into the tray and discharged. The content of each tray was passed into a 20 μm sieve to collect the nematodes.

Table 1
Pedological properties of the test soils.

Soil	pH (KCL)	OM (%)	Sand (%)	Silt (%)	Clay (%)	N_{tot} (%)	C/N	WHC (%)	CEC (Meq/100 g)	Soil type
Portugal	6.3	3.7	75.9	14.7	9.4	0.13	16.5	42.4	10.4	Loamy sand
Brazil	5.3	4.8	22.2	15.3	62.5	0.26	10.8	76.8	14.3	Clay

Codes: OM—organic matter, C/N—organic carbon/total nitrogen; WHC—water holding capacity; CEC—cation exchange capacity.

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2.2.3. Test performance

The soil previously reinoculated with the original microflora was spiked with an aqueous solution of the insecticide/nematicide Furadan 350SC (a carbofuran commercial formulation from FMC, SP, Brazil; 350 g a.i. L^{-1}). Different proportions of the stock solution were mixed in the pre-moistened soils to create the following range of dilutions: 25%, 50%, 100% and 200% of the recommended dose (RD) for sugar cane plantations (5 l/ha; ~1.167 mg a.i./kg soil DW considering a soil density of 1.5 g/cm^3 and 10 cm of incorporation depth). To the control (uncontaminated) soils only water was added.

After soil contamination, plastic boxes (7 cm Ø × 6 cm height; with perforated lids) were filled with 20 g (DW) of soil. Then, each one of the 12 or 16 replicates (6 × 2 exposure periods for treated samples or 8 × 2 exposure periods for the controls, respectively) was inoculated with 2 mL of the nematode suspension (an analogous procedure to the introduction of the test organisms in a standardized single species test), containing about 200 or 300 nematodes respectively, for the test with the Brazilian or Portuguese communities, which is within the expected range for agroecosystems (Yeates and Bongers, 1999). During the inoculation of control and spiked samples, 9 aliquots (2 mL) from each original nematode suspension (Brazilian or Portuguese) were collected at randomly chosen intervals (e.g. the first aliquot was taken after 5 successive inoculations, the second after 20 and so on) into 5 mL glass vessels and kept at 4 °C for further characterization of the initial nematode community.

The amount of solutions added per treatment (for the inoculation of microflora, moistening of the soil before pesticide spiking, carbofuran contamination and nematode inoculation) was adjusted to achieve initial moisture content of 70% or 60% of the WHC, respectively, for the Brazilian or Portuguese assays.

An additional vessel, without nematodes, was prepared for controlling pH and moisture during the test. The replicates were incubated at 23 ± 1 or 21 ± 1 °C (respectively, for Brazilian and Portuguese tests) in the dark. After 14 and 28 d of exposure (hereafter designated as 14 d or 28 d), for each treatment, 6 replicates (8 for the control) were processed, i.e., the soil was removed from the vessels to the extracting trays with a spatula; the vessels were slightly rinsed with water and the solution was added to the soil surface above the trays. The nematodes were then extracted as described in Section 2.2.2 (although smaller trays, consisting of cylindrical boxes of 10 cm Ø × 4 cm, were used), and kept at 4 °C until further nematode counting and identification (performed, at most, within 10 d).

2.2.4. Nematode identification and quantification

The first 100 individuals (or the total number, in samples containing less than 100 individuals) were spread into Doncaster plates (Doncaster, 1962) and identified to family level under an inverted microscope (100 and 200 × magnification) according to Goodey (1963) and to an Interactive Nematode Identification Key (*available at:* http://nematode.unl.edu/key/nemakey.htm). The main organs/structures used for taxonomic identification were the body cuticle, head/mouthparts, esophagus, reproductive system and tail.

Whenever doubts about the taxonomic group of a specific organism persisted, it was transferred to a glass slide, killed by heat and observed under an optical microscope (100 and 400 × magnification). Each nematode was also assigned to a trophic group (plant parasites/feeders—PLF, fungal feeders—FGF, bacterial feeders—BTF and predators/omnivores—PD–OM) according to Yeates et al. (1993). According to this author, for most soil nematode families, the organisms belonging to a specific family share the same feeding habit.

The trophic composition of the 100 identified organisms was used to extrapolate the total trophic and taxonomic composition of the respective sample.

The remaining nematodes were counted under a low magnification (40 and 100 × magnification) under an inverted microscope.

2.3. Chemical analysis

Soil samples (400 g Wet Weight/treatment) were collected and frozen for further determination of carbofuran concentrations in soil and in soil eluates.

Eluates were prepared in duplicate, following standard methods (DIN, 1984). The soil (50 g DW) was mixed with deionized water (1:10 ratio, w/v), magnetically stirred during 12 h, centrifuged (3370*g*) at room temperature, the supernatant collected and stored (4 °C; dark) until use (within 48 h). Eluates were filtered through a 0.45 μm nylon membrane (Millipore) and submitted to solid phase extraction (Discovery® DSC-18); carbofuran was eluted with 5 mL of acetonitrile and the extracts were dried. The volume of extracts was reduced in a rotavapor (40 °C), transferred to a vial and evaporated to dryness under a gentle stream of nitrogen. Extracts were kept frozen (−18 °C) and re-dissolved in mobile phase just before analysis.

Soil carbofuran extraction and analysis was based on the USEPA method 8318A (USEPA, 2007). About 2 g of soil (Wet Weight; WW) were extracted with 5 × 3 mL of acetonitrile in an ultrasonic bath and centrifuged (2000 *g*). Extracts were processed as described for eluates.

The analytical instrumentation included an HPLC Jasco model with a Rheodyne 7125 injector and a loop size of 50 μL coupled to an UV detector UV Chrom-A-Scope (BarSpec) operating between 190 and 370 nm. Acquisition was performed at 210 nm and started at 5 min until 15 min. For peak confirmation, the existence of a peak at 278 nm was checked at the specific retention time and the UV spectrum of the sample compared with the standard (Carbofuran standard, CIL Inc.) spectrum. The analytical column used was a Luna C-18 (250 × 4.6 m; 5 μm; 100 Å), with a guard column of the same material. The mobile phase selected consisted of Milli-Q water and 60% of methanol (flow rate of 0.8 mL/min).

Carbofuran standard and a stock standard solution (10 mg/L) were prepared in acetonitrile. Calibration standards were prepared by dilution of the stock solution with the mobile phase (from 0.25 to 3 mg/L). External calibration was used for quantification.

The limit of quantification (LOQ), calculated based upon an S/N ratio of 10:1, was 20 μg/kg for soil samples and 0.7 μg/L for eluates. Mean recovery was 81 ± 7% for soil samples and 94 ± 8% for eluates.

2.4. Statistical evaluation

To investigate the effects of carbofuran on total abundance (total number of organisms recovered per replicate), total abundance of each feeding group, trophic structure (relative abundance of each feeding type) and number of families, an one-way ANOVA followed by post-hoc comparisons with the control (Dunnet test) to derive No Observed Effect Concentrations (NOEC) values. Prior to ANOVA data on the above mentioned endpoints were analyzed for normality (Kolmogorov–Smirnov test) and for variance homogeneity (Levene Test). When violations of normality and/or homogeneity occurred, a log $(x+1)$ transformation was applied. Effects were considered statistically significant for p levels ≤ 0.05.

Whenever feasible, the eluate concentrations causing 50% of decline (EC50 and respective 95% confidence intervals) in the total abundance or abundance of nematode feeding types (only feasible for the Portuguese data) were calculated (as the main exposure route of nematodes to chemicals in soil is expected to be via interstitial water; Sochová et al., 2006) using non-linear regressions (EC, 2004). To eliminate the higher inter-replicate variability, the average number of nematodes per treatment was used. All analyses were performed in Statistica 7.0 (*available at* http://www.statsoft.com/).

Potential effects of carbofuran on the community structure were analyzed by Analysis of Similarity (ANOSIM), by comparing the trophic structure and family composition of the carbofuran treated samples with those of the control. Whenever significant differences were found, the Similarity of percentages (SIMPER) analysis was used to identify the families or feeding groups responsible for the observed change and their individual contribution (in terms of percentage) for the overall shift. Both ANOSIM and SIMPER analysis were ran in Primer 5.2.6 (Clarke and Gorley, 2001) using log$(x+1)$ transformed data.

3. Results

3.1. Carbofuran concentrations

The carbofuran concentrations obtained in soil samples were within the expected values for the RD used (about 1.167 mg a.i./kg soil DW, see Section 2.2.3). Soil concentrations were quite similar for Brazilian and Portuguese samples (Table 2). Based on the lower eluate concentrations determined for the Brazilian samples, the carbofuran exposure of nematodes extracted from this soil was also expected to be lower (Table 2).

Table 2
Carbofuran concentrations (mg/kg of soil DM) in the soil samples and eluates (prepared from soil and using a dilution factor of 10 ×; see Section 2.3) contaminated with four doses of Furadan, collected at the beginning of the experiment ($d=0$). RD—recommended dose.

Furadan dose (%RD)	Portugal		Brazil	
	Soil (mg/kg)	Eluates (mg/L)	Soil (mg/kg)	Eluates (mg/L)
0	bLOQ	–	bLOQ	–
25	0.315 ± 0.020	0.026 ± 0.001	0.193 ± 0.004	0.016 ± 0.002
50	0.755 ± 0.015	0.050 ± 0.002	0.514 ± 0.033	0.037 ± 0.006
100	1.302 ± 0.079	0.105 ± 0.003	1.023 ± 0.021	0.070 ± 0.002
200	2.977 ± 0.354	0.203 ± 0.006	2.432 ± 0.162	0.123 ± 0.014

bLOQ—below limit of quantification.

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3.2. Composition of the inoculated community

The inoculated nematode community from the Portuguese soil was composed by 17 families with a clear dominance of the bacterial-feeding nematodes (almost 60%) followed by plant feeders (28.3%) (Table 3). The nematode community from Brazilian soil, although sharing the majority of the families with the Portuguese community (14 in a total of 17), consisted mainly of plant feeders (77%) of the family Hoplolaimidae (72.6%), followed by bacterial feeders (13.6%) (Table 3).

3.3. Effects of carbofuran in total nematode abundance

The nematode recovery rate in the controls was approximately 25% and 30% of the initial abundance (see Table 3 for the estimated abundance of the inoculated communities), respectively, for the Brazilian and Portuguese communities, and did not varied greatly between the two exposure periods (Fig. 1). In general, a high variability was found between replicates. The results indicate a markedly different response of the two nematode communities to carbofuran contamination. At the Portuguese community, total abundance significantly decreased at the two highest concentrations, already within 14 d of exposure (One Way Anova, Dunnet test; NOEC = 0.050 mg/L; $p < 0.05$; Fig. 1 PT) when compared to the control. The estimated EC50s for the decline in total nematode abundance indicate a higher toxicity after 28 d of exposure (Table 4). At the Brazilian community, although a decrease in the average number of nematodes was found at the highest insecticide concentrations when comparing with the controls (14 d—from 45 to 25, 28 d—from 52 to 37, respectively, for the controls and at 0.123 mg/L of carbofuran), these differences were not statistically significant (One Way Anova, Dunnet test; $p > 0.05$; Fig. 1 BR).

Table 3
Composition of the inoculated nematode communities of Portugal and Brazil, expressed as relative abundance of families, allocated in four feeding groups and total abundance (sum of the organisms extracted and inoculated per replicate). Values express mean ± standard deviation and are based in the counting of 9 samples (see Section 2.2.3 for details).

	Portugal	Brazil
Relative abundance (%)		
Bacterial feeders (BTF)	**59.7 ± 4.4**	**13.6 ± 3.4**
Cephalobidae (Acrobelinae)	0.4 ± 0.5	3.0 ± 1.7
Cylindrocorporidae	0.5 ± 1.3	0.3 ± 0.5
Diplogasteridae	5.6 ± 2.6	0.7 ± 1.0
Camacolaimidae (Halaphanolaiminae)	0.7 ± 0.9	F
Monhysteridae (Prismatolaimus sp)	–	0.9 ± 1.3
Panagrolaimidae	39.3 ± 3.6	3.1 ± 3.8
Plectidae	0.2 ± 0.6	–
Rhabditidae	13.0 ± 3.1	5.6 ± 4.5
Plant parasites/feeders (PLF)	**28.3 ± 4.0**	**77.1 ± 3.7**
Criconematidae	0.1 ± 0.3	F
Heteroderidae	2.1 ± 1.0	–
Hoplolaimidae	7.9 ± 4.1	72.6 ± 4.5
Pratylenchidae	2.3 ± 1.6	0.1 ± 0.3
Tylenchidae	15.9 ± 4.1	4.4 ± 1.3
Fungal feeders (FGF)	**5.7 ± 2.8**	**2.2 ± 1.5**
Aphelenchoididae	5.5 ± 2.9	2.2 ± 1.5
Diphtherophoridae	0.2 ± 0.4	F
Predators/omnivorous (PD/OM)	**6.3 ± 1.8**	**7.1 ± 2.1**
Dorylaimidae	5.1 ± 1.7	5.3 ± 2.3
Mononchidae	1.2 ± 0.6	1.8 ± 1.2
Trichodoridae	*F*	–
Total abundance	*303.8* ± 19.8	*219.1* ± *21.5*

F—individuals found in the treated samples but not in the initial inocula.

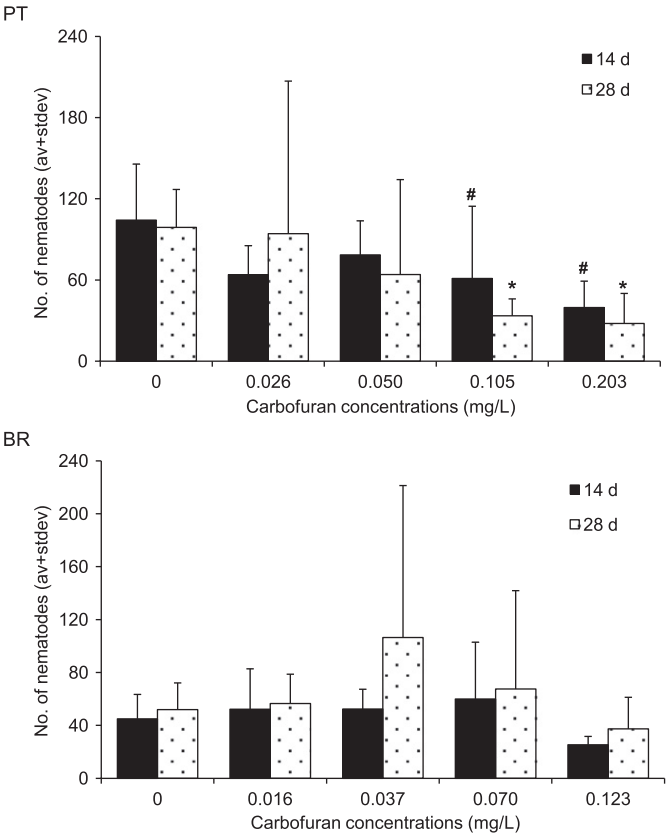

Fig. 1. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 2 for correspondences between Furadan doses and carbofuran concentrations), on total abundance (number of organisms recovered per treatment) of nematodes from Portugal (PT) and Brazil (BR), 14 and 28 d after the test start (black and white pointed bars, respectively). Values express mean ($n = 6$ or $n = 8$, respectively, for treated and control samples) ± standard deviation. *, #—statistically different from the respective controls (One Way Anova, Dunnet Test, $p < 0.05$).

Table 4
Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 2 for correspondences with Furadan doses and soil concentrations) on the decline of total abundance and abundance of feeding types of a Portuguese nematode community, exposed during 14 and 28 d. Values represent EC50 (and 95% confidence intervals). BTF—bacterial feeders; PLF—plant parasites/feeders; FGF—fungal feeders; n.d.—not determined.

	Carbofuran toxicity (mg/L)	
	14 d—EC50	28 d—EC50
Total abundance	0.153 (0.091–0.257)	0.064 (0.034–0.119)
BTF	n.d.	0.078 (0.068–0.089)
PLF	0.069 (0.043–0.112)	0.068 (0.046–0.099)
FGF	0.051 (0.029–0.089)	n.d.

3.4. Effects of carbofuran in the nematode family composition

As it was observed with nematode abundance, stronger toxic effects in the family composition were observed for the Portuguese nematode community (Fig. 2 PT). After 14 d of exposure to carbofuran contamination, a significant decrease in the number of families was detected at eluate concentrations equal to or higher than 0.105 mg/L, in comparison to the control (One Way Anova, Dunnet test; $p < 0.05$; NOEC = 0.050 mg/L; Fig. 2 PT—14 d). This toxic effect increased after 28 d as the exposure to carbofuran concentrations equal or higher than 0.050 mg/L reduced significantly the number of families found (One Way Anova, Dunnet test; $p < 0.05$; NOEC = 0.026 mg/L; Fig. 2 PT—28 d). For both

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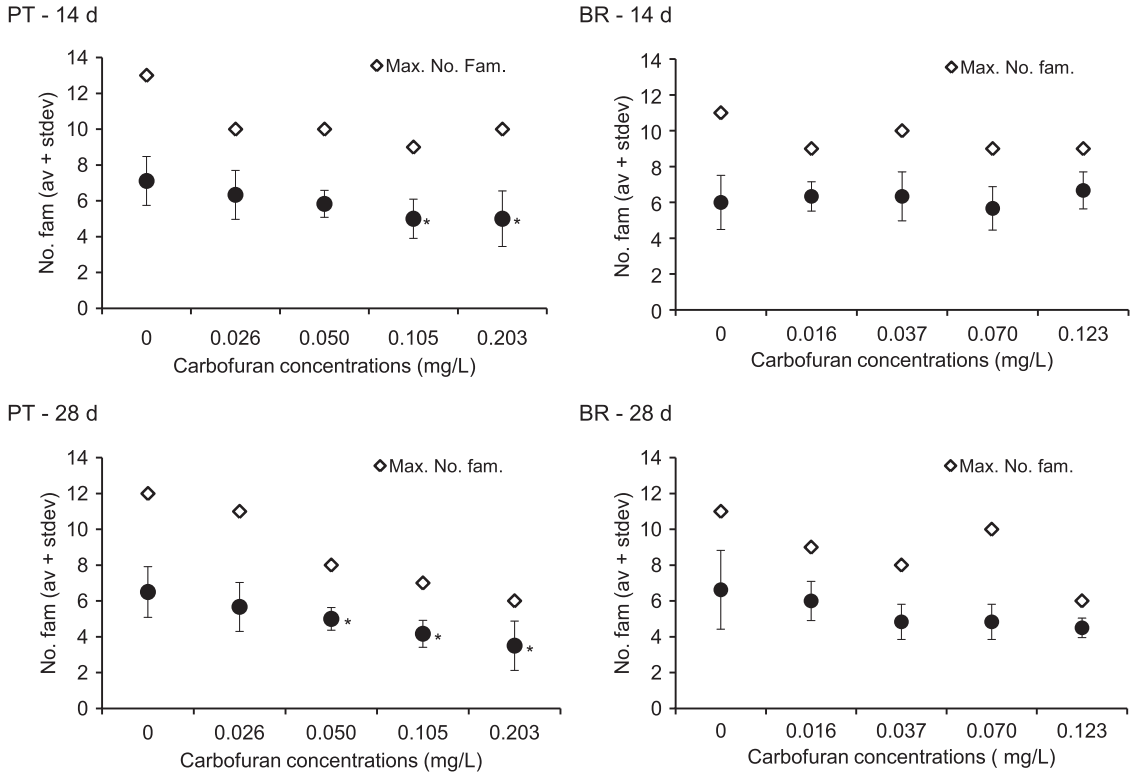

Fig. 2. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 2 for correspondences between Furadan doses and carbofuran concentrations), on the number of families found in two nematode communities from Portugal (PT) and Brazil (BR), after 14 and 28 d of exposure. Black points represent mean ($n=6$ or $n=8$, respectively, for treated and control samples ± standard deviation) values and diamonds represent the maximum number of families (max. no. fam.) found within treatments. *—mean number of families statistically different from control (One Way Anova, Dunnet Test, $p<0.05$).

exposure periods, the carbofuran soil contamination caused a significant change in the family structure in all concentrations tested (ANOSIM; $p<0.05$). Diplogasteridae, Rhabditidae, Aphelenchoididae and Tylenchidae had the major contribution to the community shifts (Fig. 3).

For the Brazilian nematode community, as observed for total abundance data, none of the carbofuran concentrations caused a significant reduction in the number of families relative to the control (One Way Anova, Dunnet test, $p>$ 0.05; NOEC $\geq$ 0.123 mg/L; Fig. 2 BR). Despite this, a decrease in the maximum number of families was found (from 11 in the control to 6 for carbofuran concentrations of 0.123 mg/L; Fig. 2 BR) after 28 d of exposure (Fig. 2 BR—28 d). Similarly, after this period, ANOSIM detected significant differences in the family structure of the Brazilian nematode community at this concentration, when compared to the control (ANOSIM, $p<0.05$). The families that contributed most to this dissimilarity were Rhabditidae, Panagrolaimidae (their abundance decreased at the highest carbofuran concentration, in comparison to the control) and Aphelenchoididae (with higher abundance at the highest carbofuran concentration than in the control), respectively, with 27%, 12% and 19% of contribution (SIMPER, $p<0.05$; data not shown).

Also, focusing on the total abundance of each nematode family in both assays, there were some families that disappeared (generally the less abundant ones) or their abundance was strongly reduced along the contamination gradient (see Appendix A).

3.5. *Effects of carbofuran in the total abundance of nematode feeding groups and global trophic structure*

In both assays, an "incubation effect" was observed since the trophic structure in the controls (after 14 and 28 d; Fig. 4) was different from the inoculated community (IC) (Table 3). In the Portuguese nematode community there was a strong increase in the bacterial feeders (from about 60% in the IC—Table 3 to more than 80% in the controls—Fig. 4 PT) accompanied by the decline of the other three trophic groups, specially the plant feeders (from about 28% in the IC to 9% and 6% in the controls, after 14 and 28 d, respectively; Table 3 and Fig. 4 PT). The same trend was observed in the controls inoculated with the Brazilian community: the proportion of plant feeders was much lower than in the IC (77% in the IC and 45% and 20%, respectively, after 14 and 28 d; Table 3 and Fig. 4 BR), while the bacterial and fungal feeders increased their relative abundance (from about 2% and 14% in the IC to about 13% and 50% in the controls, after 14 and 28 d, respectively, for BTF and FGF; Table 3 and Fig. 4 BR).

The high variability of data on the absolute abundance of each feeding group, often impaired the detection of significant differences between the different carbofuran concentrations and the control. This was especially true for the data with the Brazilian nematode community (all NOECs $\geq$ 0.123 mg/L). However, significant effects were found within the Portuguese nematode community (graphs are available in Appendix B). After 14 d, the total abundance of plant and fungal feeders was significantly lower for carbofuran concentrations of 0.105 and 0.203 mg/L, if compared to the control (One Way ANOVA, Dunnet test; $p<0.05$; NOEC= 0.050 mg/L). Estimated EC50s were lower for fungal feeders if compared to plant and bacterial feeders (Table 4). After 28 d, the same significant effect was found but for bacterial feeders (One Way ANOVA, $p<0.05$; Dunnet test; NOEC=0.050 mg/L); the abundance of plant feeders was also significantly decreased at concentrations of 0.050 and 0.203 mg/L (One Way ANOVA, Dunnet test; $p<0.05$; NOEC=0.026 mg/L). Carbofuran (eluate) concentrations of 0.068 and 0.078 mg/L were expected to cause a 50% decline in the populations of plant and bacterial feeders, respectively (Table 4).

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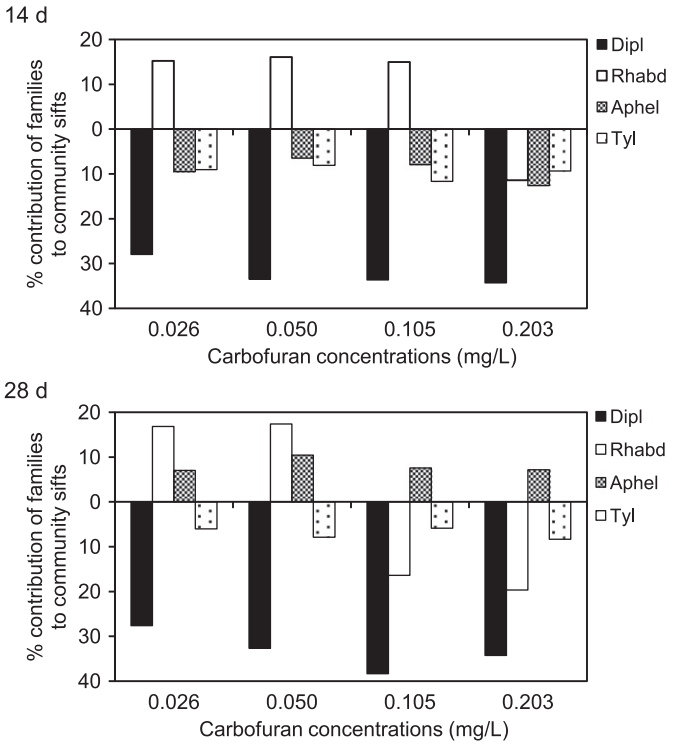

Fig. 3. Shifts in the family structure of a Portuguese nematode community induced by carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 2 for correspondences between Furadan doses and carbofuran concentrations): representation of the four families with the greatest contribution to the overall changes. Deviations above the zero line (representing the control) represent an increase in abundance along the concentration gradient while deviations below the line represent a decrease in abundance in comparison with the control. Dipl—Diplogasteridae; Rhabd—Rhabditidae; Aphel—Aphelenchoididae; Tyl—Tylenchidae.

No significant effects of carbofuran treatment in the relative abundance of each feeding group were found for both countries (One Way Anova; $p > 0.05$). Accordingly, analysis of similarity (ANOSIM) detected no significant differences in the global trophic structure neither for Portuguese nor Brazilian nematode communities. Despite this, the increase of predators–omnivores and fungal feeders in the contaminated soil from Brazil was observed, respectively, after 14 d or at both exposure periods (Fig. 4 BR); fungal feeders also increased their relative abundance in the Portuguese contaminated samples after 28 d (Fig. 4 PT—28 d).

4. Discussion

4.1. General considerations on the testing strategy

The low nematode recovery rate, obtained in the controls and in the treated soil samples, was found to be the main difficulty of the testing strategy used in the present work. Moreover, the procedural changes taken for the Portuguese assay did not produce satisfactory results given the slight increase in the nematode recovery rate (5%). This problem has been reported in previous studies involving soil inoculation with nematodes and/or extraction from uncontaminated (control) soil after short periods of time (Djigal et al., 2004; Kammenga et al., 1996; Parmelee et al., 1997; Viketoft, 2008).

Unfortunately, in soil nematode testing, one of the major challenges seems to be to find a more effective method for recovering nematodes and, at the same time, obtain an accurate assessment of the composition of nematode community as biased estimations occur independently of the method used (McSorley and Frederick, 2004, Sochovà et al., 2006 and references therein). Notwithstanding, active extraction methods based on the motion capacity of live nematodes (like the one used in the present work), seem to be preferable above others (Bell and Watson, 2001; Kammenga et al., 1996).

The extraction procedures for all samples and the inoculation of the nematode initial community (IC) probably worked as a stress factor that killed some organisms and impaired reproduction of the majority.

The low recovery rate in the inoculated samples was also followed by a shift in the community trophic structure of the controls from both countries, when compared to the IC. These facts suggest that these shifts were somehow more pronounced than the ones induced by carbofuran contamination, for the same endpoint.

Considering the total abundance of each one of the feeding groups in the control and in carbofuran treated samples, after both 14 and 28 d of exposure, the BTF nematodes generally increased while a decline in the PLF was observed (Fig. 4; Appendix A). This was somehow expected since there was no particular food supply to plant feeders, contrasting with the pre-inoculation of soil with the original microflora that probably led to increased food availability for bacterial feeders. The rate of BTF increase after 14 d, when compared to the IC, was approximately of 30% in both Portuguese and Brazilian assays, suggesting that the extension of the incubation period for the microflora in the Portuguese assay did not greatly influence the results. Furthermore, it is likely that the defined exposure periods would only allow the reproduction of some opportunistic bacterial feeding nematodes while for the other groups only lethal effects could be observed. Indeed, life span of nematodes can be as short as one week to opportunistic bacterial feeders, 4–8 weeks for plant feeders until several years for large plant feeders and predators/omnivores under undisturbed conditions (Ferris, 2004). Thus, for further validation of the testing strategy, improvements are needed, namely extending the exposure periods (to, at least, two months), provide food to plant feeders (e.g. by sowing a seed in each replicate) and extending the final extraction period to 96 h (McSorley and Frederick, 2004), besides testing of other nematode communities.

However, the reported drawbacks were expected and much probably constitute associated risks of introducing ecological realism in the testing strategy. The use of nematode communities, consisting of several unidentified species, for which optimal ecological requirements (e.g. food, temperature and moisture) are unknown, is surely associated with increased uncertainty/variability of data, if compared with traditional standardized single species testing (ASTM, 2008; ISO, 2009).

Summarizing, despite the reported drawbacks and the improvements needed, the testing strategy showed to be valid as a starting point to promote the testing of nematode communities under laboratory conditions in ecotoxicology related-studies. Moreover, some of the problems reported in Section 1 for field and semi-field studies could presumably be diminished trough the laboratorial exposure of nematodes, generating data with lower variability associated and requiring, at the same time, less space, time and costs.

4.2. Toxicity of carbofuran to the nematode communities

The two nematode communities responded similarly to carbofuran contamination, although the Portuguese community was clearly the most negatively affected. Some possible explanations could be the moderately higher carbofuran concentrations in the Portuguese samples (in average, $\approx$ 27% higher for soil and 34% for eluate samples; Table 2) or a higher intrinsic sensitivity of

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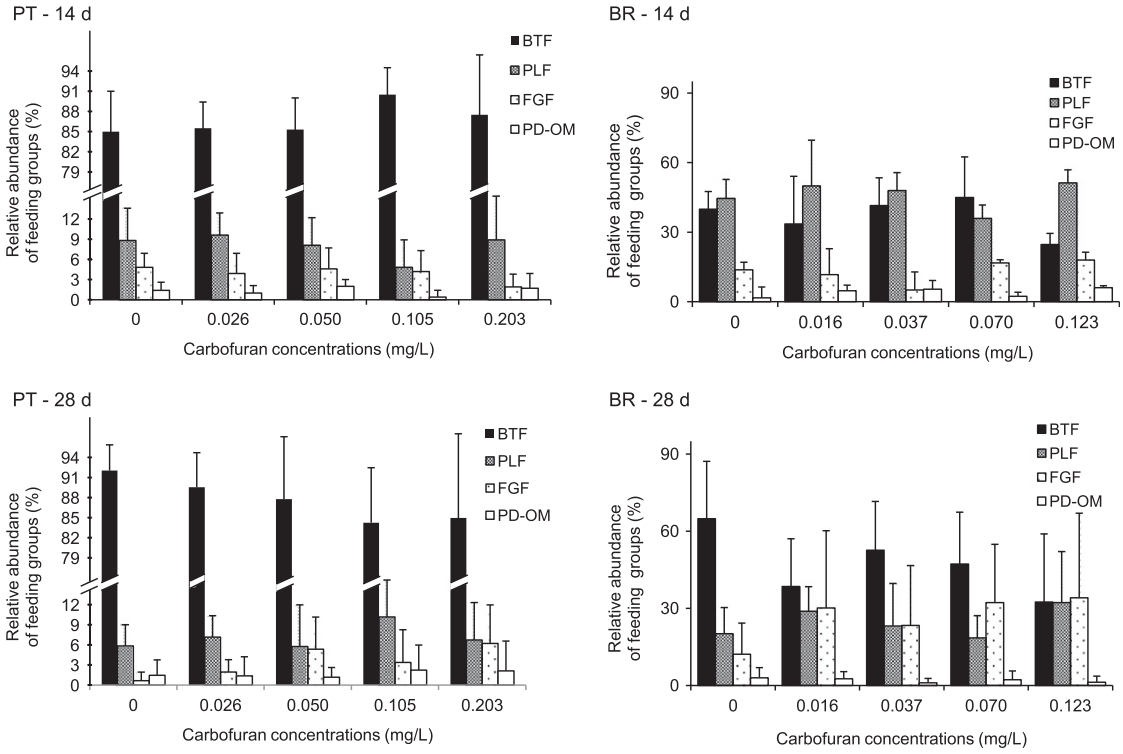

Fig. 4. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil (see Table 2 for correspondences between Furadan doses and carbofuran concentrations), on the trophic structure of two nematode communities from Portugal (PT) and Brazil (BR), after 14 and 28 d of exposure. Vertical bars represent mean ($n=6$ or $n=8$, respectively, for treated or control samples ± standard deviation) values of relative abundance of four feeding groups. BTF—bacterial feeders; PLF—plant parasites/feeders; FGF—fungal feeders; PD/OM—predators/omnivores.

Portuguese nematodes to pesticides (not investigated). However, the critical factors determining the different responses of the organisms have, much probably, been the soil properties, namely the clay and organic matter contents. Carbofuran soil adsorption positively correlates with both clay and organic matter contents (Singh and Srivastava, 2009; Weber et al., 2004). Since the last two were clearly higher in the Brazilian soil ((62.5% and 4.8%); Table 1), a stronger adsorption of carbofuran was expected, resulting in a lower available fraction in the soil pore water (main exposure route for soil nematodes). Accordingly, the decreased efficacy of nematicides in clayed soil, when compared with sandy soils, has been previously reported (Araya, 2003, Bond et al., 2000). Also, a higher moisture level (as in the Brazilian soil) seems to favor the carbofuran biodegradation (Shelton and Parkin, 1991) lowering the possible toxic effects.

4.2.1. *Effects on the total nematode abundance*

Toxic effects of carbofuran on the total abundance of nematodes were only registered within the Portuguese community, at the two highest carbofuran concentrations, with more pronounced effects after 28 d of exposure (Fig. 1).

Comparisons with other data are difficult since the available information on the effects of pesticide pollution (mainly nematicides and insecticides) on total nematode abundance respects to field or semi-field studies. Even though, in some cases, decreased abundance was observed in treated soils (Pen-Mouratov and Steinberger, 2005; Yardim and Edwards, 1998) while for the majority, no negative effects were found (Coleman et al., 1994; Griffiths et al., 2006; Moser et al., 2004; Parmelee et al., 1997; Wada and Toyota, 2008). This might be related with the larger variance associated to experiments carried out of the laboratory context (Sochova et al., 2007). Moreover, nematicides are not expected to have direct lethal effects on target nematodes, but to limit their mobility and thus the ability to infect the plant hosts (Wright and Womack, 1981) as it is the case for carbofuran, an acetylcholine esterase (AChE) inhibitor (IUPAC, 2010). Despite the low persistency of carbofuran in soils (typical DT50 of 29 d; IUPAC, 2010), it is possible that the chronic exposure of nematodes might have boosted its toxicity and delayed the recovery of AChE activity, as it was reported for earthworms (Panda and Sahu, 2004). Also, the presence of metabolites resultant of carbofuran degradation (e.g. 3-hydroxy-carbofuran and 3-keto-carbofuran) might have caused toxicity, like it was reported for *Meloidogyne incognita* (Nordmeyer and Dickson, 1990).

Data from single species tests with other soil invertebrates and carbofuran suggest that the sensitivity of nematode communities to this insecticide might be similar to that of earthworms. The comparable sensitivity of nematode single species tests with enchytraeids, earthworms or springtails tests has been previously suggested (see Sochová et al., 2006 and references therein).

For instance, in the present study, carbofuran soil concentrations of 1.3 and 2.9 mg/kg (0.105 and 0.203 mg/L were found in soil eluates, respectively; Table 2) had significant hazard effects on nematode abundance in the Portuguese assay (Fig. 1 PT). These are within the range of lethal concentrations causing 50% mortality (LC50s) in *Eisenia andrei* in artificial soil (5–10 mg/kg), reported by Van Gestel (1992). Recently, exposing the same earthworm species to the same pesticide in three artificial soils, De Silva and van Gestel (2009) estimated slightly higher LC50 values ($\approx$ 12 mg/kg) but median effects on reproduction (EC50s) were one order of magnitude lower ($\approx$ 1 mg/kg). The results obtained in the present study are somehow in agreement given that the estimated EC50s ranged between 0.064 (28 d) and 0.153 (14 d) mg/L (Table 4); these roughly correspond to soil concentrations from 0.6 to 1.5 mg/kg (only considering the dilution factor of 10 × used for eluate preparation; see Section 2.3).

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4.2.2. Effects on the nematode family composition

The decrease in the number of families found in the carbofuran treated samples (Fig. 2), especially within the Portuguese community, showed that there was a loss of diversity due to insecticide contamination. The significant shifts detected in the family structure, at all carbofuran treatments in the Portuguese nematode community, and at the highest carbofuran concentration after 28 d, in the Brazilian community, were due mainly to the general decrease in relative abundance of most families along the treatments, that, in some cases, reached zero values (Appendix A). The reduction in the number of nematode taxa after exposure to the fungicide carbendazim has already been reported (Moser et al., 2004).

However, for both Portuguese and Brazilian assays, there was a strong increase in the number of Rhabditidae and Panagrolaimidae in some replicates, which contributed to the high variability found in the nematode abundance (especially at the doses 25%, 50% and 100%RD; see Appendix A and Table 2 for correspondences with carbofuran concentrations). Members of these families are classified as extreme opportunists and rapid colonizers, having explosive growth patterns under high microbial activity (Bongers, 1999). One cannot exclude that these observations might have been an artifact of the test system. Indeed, previous inoculation of soil with the native microflora together with the disappearance or decrease in abundance of other families most vulnerable to carbofuran contamination might have increased food availability for these opportunistic families, that rapidly increased their numbers.

4.2.3. Effects on the total abundance of nematode feeding groups and global trophic structure

The soil contamination by carbofuran caused statistically significant shifts in the total abundance of nematode feeding groups. This occurred on the Portuguese nematode community only, where a general decrease in the abundance of most feeding groups was observed.

The abundance of the dominant group, the bacterial feeders (BTF), significantly decreased after four weeks of exposure only at the two highest carbofuran concentrations. The existence of higher carbofuran concentrations during the first two weeks may have stimulated the growth of microbial populations (Lo, 2010), leading to more food availability and reducing the potential hazard effects of carbofuran to this group of organisms. However, a significant reduction of bacterial feeding nematodes following the in situ contamination of a semi-arid grassland soil with carbofuran was reported by Ingham et al. (1986). The application of other nematicides and insecticides under field conditions also caused a significant decrease in BTF nematodes (Pen-Mouratov and Steinberger, 2005; Yardim and Edwards, 1998).

A lower number of plant feeding nematodes (PLF) was recovered at the highest carbofuran concentrations in both exposure periods indicating a toxic effect of this insecticide. However, contradictory information has been reported since there were cases where insecticide applications had stimulatory effects to this feeding group (Yardim and Edwards, 1998) and others where the opposite response occurred (Parmelee et al., 1997; Pen-Mouratov and Steinberger, 2005).

The fungal feeders (FGF) were affected by carbofuran contamination but opposite responses were observed at the two exposure periods. If 14 d of exposure to carbofuran caused significant toxic effects at the two highest carbofuran concentrations, the longer exposure caused a slight increment in the abundance of these organisms (although not significant). These observations might indicate direct toxic effects of the insecticide over the nematodes or lower food availability during the first period. Our results somehow conflict with other data since short-term studies (one week) on the effects of the insecticide malathion on FGF nematodes in a semi-field microcosm system did not cause toxic effects (Parmelee et al., 1997), while in longer studies (from one month to one year), a lower abundance of FGF was found in insecticide treated samples (Pen-Mouratov and Steinberger, 2005; Yardim and Edwards, 1998).

Predators–omnivores, the less abundant group on both Portuguese and Brazilian assays (Appendix A), are known to be indicators of soil disturbance (Bongers and Bongers, 1998; Moser et al., 2004) and sensitive to nematicides (Smolik, 1983) and other insecticides (Yardim and Edwards, 1998). However, in the present study no significant effects were found perhaps due to the low number of organisms recovered. Indeed, in the cited experiments, performed under field conditions, the number of recovered predators–omnivores was substantially higher (Smolik, 1983; Yardim and Edwards, 1998).

Despite the decrease in the number of families and in the abundance of most feeding groups, the relative contribution (percentage) of each feeding group to the global trophic structure was not significantly affected by the insecticide contamination in any carbofuran treatment, in both Portuguese and Brazilian assays. This last endpoint seemed to be a conservative trait, i.e., the disappearance (or strong decline in abundance) of some nematode families (frequently, the less abundant), with consequent shifts in the community composition, was not closely followed by a functional shift.

However, the observed functional stability does not necessarily indicate that these nematode communities are highly resilient to pesticide disturbances as significant shifts in other endpoints (reported above) were detected.

The lack of effects also means that the disappearance (or decrease in abundance) of nematodes from a certain family was either replaced by an increase of another family within the same feeding group or accompanied by a general decrease in abundance of all feeding groups. This functional redundancy (the replacement of lost species by others with similar traits) was pointed as a weakness of trait-based approaches (Van den Brink et al., 2011). Although gaining in ecological realism, the resolution of the trait-based approach used in the present study was somehow lower than the traditional taxonomic approach. Indeed, as one nematode family usually represents a single feeding type, changes in the feeding structure can be also detected besides the (“traditional”) changes in the family structure. However, the opposite scenario is not true since changes in the feeding structure do not necessarily represent changes in the family structure, e.g. if the effects of a certain chemical mainly comprise the decrease in the species numbers of the most abundant families.

Notwithstanding, confirmation is needed with further tests with other pesticides/chemicals and nematode communities.

It is difficult to compare our results with literature data, even restricting comparisons to studies where the effects of pesticides and metals on nematode communities were evaluated. All of them were performed under field or semi-field conditions, with different soil types/land-uses and, consequently, with dissimilar nematode communities. Therefore, direct comparisons have always a high degree of uncertainty associated. For example, in most cases, the percentage of each feeding group in the whole community is not presented and the analyses were performed using the absolute abundance of each group. Indeed, significant changes in the absolute abundance of at least one nematode feeding group, as a consequence of soil contamination, have been described (Korthals et al., 1996; Parmelee et al., 1997; Pen-Mouratov and Steinberger, 2005; Yardim and Edwards, 1998). The experiments conducted by Moser et al. (2004) constitute an exception given that the relative abundance of nematodes was

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used to describe the hazard effects of the fungicide carbendazim and a significant decrease in the percentage of predators and omnivores was reported.

5. Conclusions

Although some methodological aspects need to be improved (especially the extraction efficacy), our results revealed that the use of soil nematode communities as sensitive indicators of toxic effects of pesticides is promising. A higher toxicity was observed for the Portuguese nematode community but the patterns of response of both communities were similar. The lack of significant effects for most parameters within the Brazilian nematode community was probably due to the higher clay and organic matter contents of soil that caused a lower carbofuran bioavailability but also to the high variability found among replicates.

The two major response patterns of nematode communities to carbofuran soil contamination were the decrease in the total nematode abundance and the reduction in the number of families. Significant shifts in the family composition were detected mainly within the Portuguese community (for the Brazilian, only significant shifts in the family composition were detected after 28 d, at the highest carbofuran concentration). However, the proportion of each feeding group in the trophic structure of the community did not significantly change with the different insecticide dosages. Thus, for this particular study, the trait-based approach used was not powerful enough to reveal the hazard effects of carbofuran in the trophic structure. Most probably, for this specific pesticide nematode community responses do not comprise changes in this endpoint but rather a general decrease in the abundance of all feeding groups.

The use of such a (feeding) trait-based approach is advantageous since effects can be evaluated without the need of identification at the species level. Moreover, response patterns of communities/populations are much more ecologically relevant than information from single species testing. However, an important drawback of using only this approach is the fact that possible losses of biodiversity (disappearance of some taxa, like it was observed in the present study using the taxonomic approach) cannot be observed or predicted. Thus, in nematode community ecotoxicological testing (as well as in other community related-studies), the implementation of feeding trait-based approaches as an alternative to the taxonomic characterization requires further evaluation, and depending on the objectives defined, it is recommended to consider the advantages of integrate both approaches (Van den Brink et al., 2011).

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The authors declare that the experiments presented in this work have been conducted according to national and institutional guidelines for the protection of human subjects and animal welfare.

Table A1
Family composition of the nematode communities of Portugal (PT) and Brazil (BR) (expressed as total abundance of the nematode families, i.e., the sum of organisms extracted per family per treatment) exposed to soil contaminated with four doses of Furadan during 14 and 28 d. BTF—bacterial feeders; PLF—plant feeders; FGF—fungal feeders; PD–OM—predators/omnivores; RD—recommended dose. IC—initial community. Pan—Panagrolaimidae, Rhab—Rhabditidae; Dipl—Diplogasteridae; Ceph—Cephalobidae (Acrobelinae); Cyl—Cylindrocorporidae; Cam—Camacolaimidae (Halaphanolaiminae); Monyst—Monhysteridae (Prismatolaimus sp); Plect—Plectidae; Hopl—Hoplolaimidae; Tyl—Tylenchidae ; Praty—Pratylenchidae; Het—Heteroderidae; Cric—Criconematidae; Aph—Aphelenchoididae; Dipt—Diphtherophoridae; Doryl—Dorylaimidae, Mon—Mononchidae; Trich—Trichodoridae.

	BTF								PLF				FGF			PD–OM		
Treatments (%RD)	*Pan*	*Rhab*	*Dipl*	*Ceph*	*Cyl*	*Cam*	*Monyst*	*Plect*	Hopl	Tyl	Praty	Het	*Cric*	*Aph*	*Dipt*	Doryl	Mon	Trich
PT																		
IC	*1187*	*393*	*169*	*12*	*15*	*21*	*0*	*6*	239	480	70	63	3	*166*	*6*	154	36	0
14 d																		
0	*402*	*94*	*305*	*1*	*0*	*1*	*0*	*0*	3	55	13	6	0	*41*	*1*	12	3	0
25	*192*	*108*	*27*	*0*	*0*	*0*	*0*	*0*	4	23	6	3	0	*15*	*0*	3	1	0
50	*254*	*142*	*6*	*0*	*0*	*0*	*0*	*0*	2	30	3	3	0	*23*	*0*	3	4	0
100	*146*	*189*	*2*	*0*	*0*	*0*	*0*	*0*	1	10	3	2	0	*12*	*0*	2	0	0
200	*170*	*41*	*1*	*0*	*0*	*0*	*0*	*0*	1	14	1	2	0	*4*	*0*	1	2	0
28 d																		
0	*305*	*112*	*309*	*1*	*0*	*6*	*0*	*0*	0	30	6	5	0	*5*	*0*	7	3	1
25	*205*	*150*	*113*	*0*	*0*	*59*	*0*	*0*	1	22	2	3	0	*5*	*0*	4	1	0
50	*188*	*158*	*4*	*0*	*0*	*0*	*0*	*0*	0	8	1	1	0	*19*	*0*	4	0	0
100	*156*	*16*	*1*	*0*	*0*	*0*	*0*	*0*	0	17	0	1	0	*7*	*0*	2	0	0
200	*118*	*29*	*0*	*0*	*0*	*0*	*0*	*0*	0	8	0	0	0	*8*	*1*	3	0	0
BR																		
IC	*61*	*109*	*13*	*59*	*7*	*0*	*18*	*0*	1430	88	2	0	0	*44*	*0*	105	35	0
14 d																		
0	*46*	*105*	*5*	*2*	*1*	*0*	*1*	*0*	119	22	0	0	0	*52*	*0*	6	1	0
25	*54*	*83*	*0*	*1*	*0*	*0*	*0*	*0*	113	17	1	0	0	*33*	*0*	6	5	0
50	*46*	*64*	*12*	*1*	*0*	*22*	*0*	*0*	130	9	0	0	0	*15*	*0*	12	3	0
100	*31*	*194*	*2*	*2*	*0*	*0*	*0*	*0*	82	11	0	0	0	*34*	*0*	3	1	0
200	*21*	*14*	*0*	*3*	*0*	*0*	*0*	*0*	69	8	1	0	0	*28*	*0*	4	4	0
28 d																		
0	*95*	*116*	*1*	*8*	*0*	*0*	*10*	*0*	55	19	0	0	1	*102*	*0*	6	3	0
25	*97*	*12*	*0*	*0*	*0*	*0*	*2*	*0*	75	9	5	0	0	*132*	*0*	4	3	0
50	*95*	*261*	*0*	*1*	*0*	*0*	*0*	*0*	66	2	0	0	0	*206*	*1*	8	0	0
100	*83*	*82*	*6*	*1*	*0*	*0*	*0*	*0*	50	1	0	0	1	*177*	*0*	3	1	0
200	*50*	*7*	*0*	*0*	*0*	*0*	*0*	*0*	45	9	0	0	0	*112*	*0*	2	0	0

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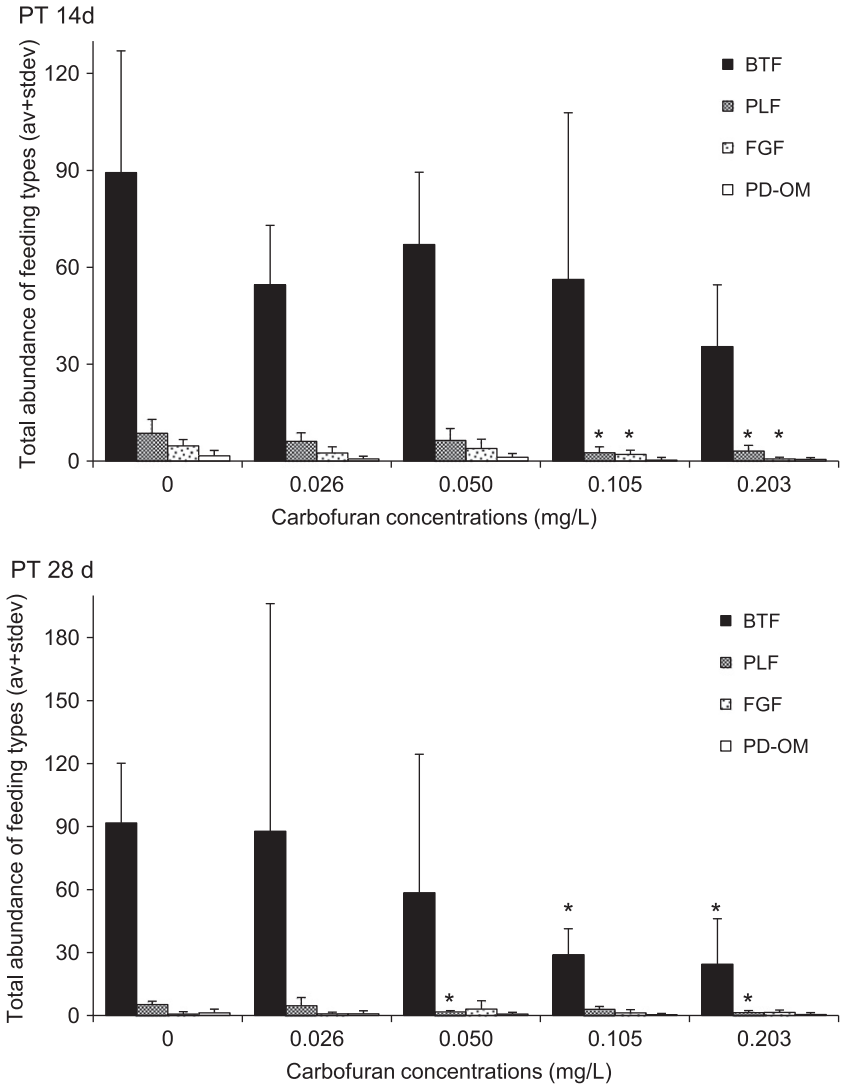

Fig. B1. Effects of carbofuran concentrations in the eluates (mg/L), prepared from contaminated soil on the total abundance of four feeding groups of a nematode community from Portugal (PT), after 14 and 28 d of exposure. Vertical bars represent mean ($n=6$ or $n=8$, respectively, for treated or control samples $\pm$ standard deviation) values. BTF—bacterial feeders; PLF—plant feeders; FGF—fungal feeders; PD/OM—predators/omnivores. See Table 2 for correspondences between Furadan doses and carbofuran concentrations. *—statistically different from the respective feeding group in the control (One Way Anova, Dunnet Test, $p < 0.05$).

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Appendix A

See Table A1.

Appendix B

See Fig. B1.

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