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Original article

Chronic ecotoxicity of ciprofloxacin exposure on taxonomic diversity of a meiobenthic nematode community in microcosm experiments

Ahmed Nasri^{a,*}, Amel Hannachi^a, Mohamed Allouche^a, Badreddine Barhoumi^b, Ibtihel Saidi^a, Mohamed Dallali^a, Abdel Halim Harrath^{c,d}, Lamjed Mansour^c, Ezzeddine Mahmoudi^a, Hamouda Beyrem^a, Fehmi Boufahja^a^aLaboratory of Environment Biomonitoring, University of Carthage, Faculty of Sciences of Bizerta (FSB), 7021 Zarzouna, Bizerta, Tunisia^bLaboratory of Hetero-Organic Compounds and Nanostructured Materials (LR18ES11), Department of Chemistry, Faculty of Sciences of Bizerte, University of Carthage, 7021 Zarzouna, Tunisia^cKing Saud University, Zoology Department, College of Science, Box 2455, Riyadh 11451, Saudi Arabia^dUniversity of Tunis El Manar, Higher Institute of Applied Biological Sciences of Tunis, 2092 Tunis, Tunisia

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ABSTRACT

A laboratory bioassay was performed to assess the ecotoxicity of ciprofloxacin on a meiobenthic nematodes community from Bizerte lagoon (NE Tunisia). Four ciprofloxacin doses [D1 (50 µg/g), D2 (100 µg/g), D3 (200 µg/g), and D4 (500 µg/g)] were applied to the substrate, and responses were studied after one month. Discernible differences were observed between control assemblages and those populating ciprofloxacin treated substrates. All univariate indices were affected significantly compared to those in the control microcosm with increasing antibiotic concentration. The non-parametric Multi-Dimensional Scaling based on species abundances (MDS) showed significant separation of the control microcosm from the antibiotic-treated populations. The nematode species responses to the fluoroquinolone treatments varied: *Odontophora villoti* was reduced at all concentrations of ciprofloxacin and was considered “sensitive,” whereas *Metoncholaimus pristiurus* was affected by moderate concentrations; its abundance increased with the highest dose D4 and was described as “opportunistic.” *Paramonohystera pilosa*, whose abundance increased with antibiotic doses appeared “resistant.”

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1. Introduction

Antibiotics were recently considered the second highest found category (15%) of drugs in the water plans (Santos et al., 2010). Their loads have been evaluated in hospital and wastewater treatment plant (WWTP) (Santos et al., 2009; Seifrtová et al., 2008), runoff water (Pena et al., 2007), and groundwater (Kummerer, 2003) and ranged between ng/L and µg/L levels, indicating their hard

degradation at Sewage Treatment Plants (STPs). In return, their ecotoxicity for non-target taxa have been rarely explored (Santos et al., 2010).

Antibiotics are extensively used for health applications of human beings and animals. Since several antibiotics are not fully metabolized, they are often detected in downstream outfluxes from most of wastewater treatment installations and animal facilities (Kolpin et al., 2002). Antibiotics pose a potential threat to biota and linked environmental processes in relation with the capital role bacteria play in these ecosystems. Data of Golet et al. (2002) and Kolpin et al. (2002) showed that ciprofloxacin could be found in runoff water in the United States of America and Europe at loads ranging from 0.01 to 0.03 mg/L. The acute toxicity of this chemical has been reported too for some aquatic organisms by Robinson et al. (2005). On the contrary, rare publications were devoted to the relationships of antibiotics and cyanobacteria by giving a concentration variety of mg/L (Kolar et al., 2014; Vázquez-Martínez et al., 2004).

Abbreviations: C, Concentration; H, Shannon index; d, Margalef's species richness; J, Pielou's evenness; S, species number.

* Corresponding author.

E-mail address: ahmed.nasri@fsb.u-carthage.tn (A. Nasri).

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Studies focusing on the impact of fluoroquinolone antibiotics on assemblages from Mediterranean are rare. No laboratory bioassay evaluated the effects of such antibiotics on benthic taxa. Free-living marine nematodes represent excellent tools in environmental monitoring programs (Coull and Chandler, 1992). These invertebrates distinguish themselves by their short lifecycles (days to weeks in most cases), extremely high abundances, and absence of planktonic juvenile stages (Austen and McEvoy, 1997). These small invertebrates (length ranged typically from 400 μm to 1 mm), with short generation time could be maintained with little effort in the laboratory and, thus, their ability to assess rapid responses to environmental changes is extremely promising (Mahmoudi et al., 2005; Gyedu-Ababio and Baird, 2006; Semprucci et al., 2015). As result, it is comparatively easy to keep nematodes in laboratory conditions and manipulate natural sediments in simple and non-costly experimental designs (Millward et al., 2004; Schratzberger et al., 2002). The main goals of the current work were to assess the numerical and taxonomic responses of a nematofauna after its exposure to a control and ciprofloxacin treatments.

2. Materials and methods

2.1. Sample collection and ciprofloxacin-enriched sediments

In Bizerte lagoon (NE, Tunisia), sediments and inhabiting meiofauna were collected from an offshore site (37° 13'33" N; 9° 49'24" E) using Plexiglas hand-cores with an internal diameter of 3.2 cm (for a depth equal to 10 cm). On sampling day, water depth and salinity were measured (50 cm and 37 PSU, respectively).

Freezing of sediments at $-20\text{ }^{\circ}\text{C}$ (12 h) followed by thawing at laboratory temperature (48 h) were repeated three times before being spiked with ciprofloxacin (Schratzberger et al., 2002). Then, large substrate particles ($>63\text{ }\mu\text{m}$) were removed through wet sieving and appropriate amounts of ciprofloxacin were finally added to sediment sub-samples of 100 g (dry weight; dw) to prepare four pre-fixed ciprofloxacin concentrations (in dw), D1 (50 $\mu\text{g/g}$), D2 (100 $\mu\text{g/g}$), D3 (200 $\mu\text{g/g}$), D4 (500 $\mu\text{g/g}$), once they are gently mixed with an amount of 200 g of fresh sediment.

2.2. Experimental designs and sample handling

Experiment enclosures consisted of glass bottles of 570 ml. Three replicated five treatments (one control and four enriched with ciprofloxacin) were set up (Schratzberger and Warwick, 1998). All microcosms, enriched or not with ciprofloxacin, were filled with 300 g dw and a litre of pre-filtered (1 μm) seawater collected on the sampling day of meiofauna (Schratzberger and Warwick, 1998).

Technically, each experimental enclosure was connected to an aeration pump during the exposure duration of one month and water parameters (namely, salinity, pH, dissolved oxygen and temperature) were monitored at short regular intervals of two days. At the end of the bioassay, all sediment samples were conserved in 4% neutralized formaldehyde.

Meiobenthic organisms were isolated based on the resuspension-decantation method followed by their coloring with Rose-Bengal (0.2 g/L) according to Wieser (1960). After 2 days, stained nematodes were counted and picked-up using a stereomicroscope to assess the nematode abundance. Pictorial keys (Platt et al., 2015), records and descriptions of taxa to the lowest level acquired from the word database of nematodes 'NeMys' maintained by nematologists of Ghent University, Belgium (Vanaverbeke et al., 2015), were used to identify the genus or species of free-living nematodes.

2.3. Data analysis

All data analyses were performed with the software Primer v5.0 as proposed by Clarke (1993) and Clarke et al. (2014). In details, the non-parametric Multi-Dimensional Scaling (MDS) analysis was applied using Bray-Curtis index of similarity (square-root transformed abundance) to detect whether the exposed nematode communities to ciprofloxacin became significantly different from controls in function of the contamination levels.

Discernible differences between assemblages exposed or not to ciprofloxacin, were determined by pairwise ANalysis Of SIMilarities (ANOSIM). The contribution of individual species towards average dissimilarities was deduced from the SIMPER analysis (SIMilarity PERcentages). Five univariate indices, namely, abundance, species number (S), Shannon diversity index (H'), Margalef's species richness (d), Pielou's evenness (J'), were computed. Analysis of variance (1-ANOVA) was conducted for global search of differences, and Tukey's HSD test was finally considered to compare treatment and control groups. An α of 0.05 assesses continuously the significance of differences.

3. Results

Univariate indices showed significant variation compared with controls. Measures of nematode assemblages between controls and treatment groups are shown in a graphical summary (Fig. 1). ANOVA results and multiple pairwise comparisons (Tukey HSD test) revealed discernible differences between control nematode assemblages and those collected from ciprofloxacin amended sediments, practically for all univariate indices considered ($p < 0.05$). Total nematode abundance, number of species, species richness, and Shannon-Weaver index declined significantly with an intensification in the ciprofloxacin exposure, with the exception of a significant increase in equitability (J') with the two lower doses, D2 and D3, of ciprofloxacin.

The MDS ordination showed that all treated microcosm replicates, except those contaminated with the lowest dose D1, were distinct from the controls; those of the highest ciprofloxacin dose D4 were placed at the extreme limit of the 2D-plot (Fig. 2). This pattern was confirmed by results of SIMPER analysis where an enhance in average dissimilarity was recorded between nematode communities inhabiting microcosms gradually enriched with ciprofloxacin in comparison with the control ones, even though ANOSIM probabilities did not reached the significance level ($p \geq 0.05$). The maximum average divergence in terms of community structure (44.06%) was noted between control replicates and those spiked with the uppermost concentration of ciprofloxacin (D4) (Table 1).

Seven nematode species were dominant in the control samples (C), and included *Odontophora villoti* (23.71%), *Paracomesoma dubium* (21.49%), *Metoncholaimus pristiurus* (18.15%), *Terschellengia longicaudata* (11.85%), *Paramonohystera pilosa* (7.78%), *Synonchiella edax* (3.70%), and *Daptonema trabeculosum* (3.70%). The replicates related to the lowest concentration of ciprofloxacin, D1, were populated by *Paracomesoma dubium* (20.35%), *Metoncholaimus pristiurus* (19.03%), *Odontophora villoti* (18.14%), *Paramonohystera pilosa* (12.83%), *Terschellengia longicaudata* (12.39%), *Daptonema trabeculosum* (4.43%), and *Synonchiella edax* (3.1%). At D2, 8 species including *Paracomesoma dubium* (18.29%), *Terschellengia longicaudata* (17.9%), *Metoncholaimus pristiurus* (15.73%), *Paramonohystera pilosa* (13.09%), *Synonchiella edax* (8.86%), *Daptonema trabeculosum* (8.46%), *Odontophora villoti* (5.55%), and *Metalinhomoeus tososus* (5.51%) dominated the microcosm. Eight taxa were remarkably present into microcosms spiked with D3, *Paramonohystera pilosa* (19.43%), *Paracomesoma dubium* (19.43%), *Terschellengia*

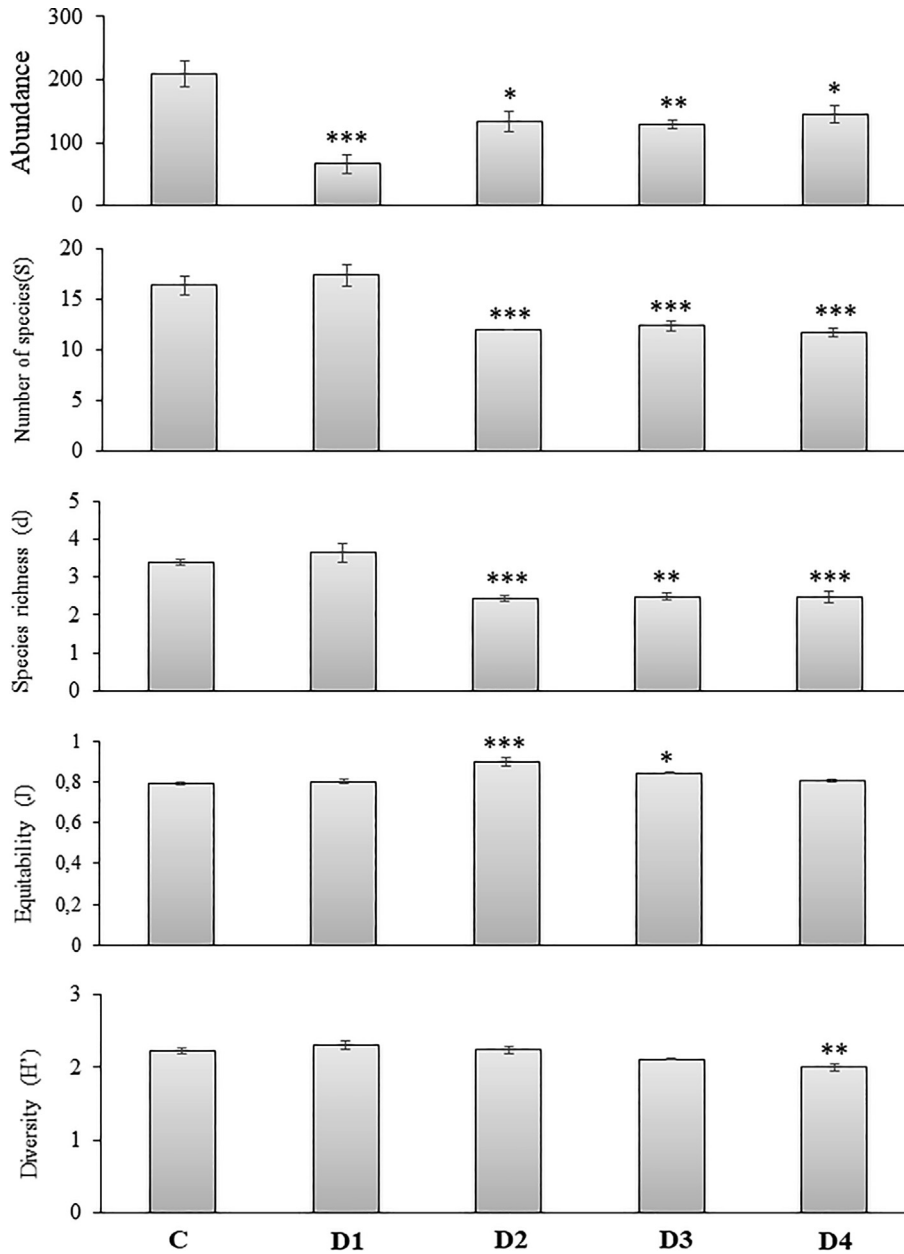


Fig. 1. Graphical summary of means and 95% pooled confidence intervals of univariate indices for nematode assemblages from each microcosm. Index of Shannon–Weaver (H'), species richness = Margalef's (d), evenness = Pielou's (J), no. species = number of species (S). Asterisk indicates a significant difference ($p < 0.05$) of the univariate measure in the contaminated microcosm when compared to the control.

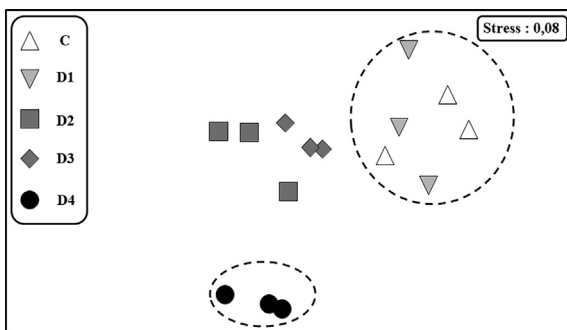


Fig. 2. Non-metric MDS ordination of square-root transformed nematode species abundance data from uncontaminated sediment control microcosm (C) and ciprofloxacin amended sediment treatments [D1, D2, D3, and D4]

Table 1

Anosim results (R statistic and significance level) and Average Dissimilarity to determine the significant differences between nematode assemblages in different treatments compared to the control and the contribution of nematode species to the dissimilarity, respectively.

Groups	R value	Average Dissimilarity (%)	Significance level
C-D1	0.856	16.4	0.1
C-D2	0.856	31.32	0.1
C-D3	0.856	27.45	0.1
C-D4	0.856	44.06	0.1

longicaudata (18.27%), *Metalinhomoeus torosus* (12.95%), *Odonotophora villoti* (9.72%), *Synonchiella edax* (5.64%), *Daptonema trabeculosum* (3.64%), and *Anticoma acuminata* (3.64%). Regarding replicates highly contaminated with ciprofloxacin D4, 7 taxa were

regularly inventoried, namely, *Metoncholaimus pristiurus* (39%), *Paramonohystera pilosa* (19.51%), *Synonchiella edax* (11.32%), *Daptonema trabeculosum* (8.17%), *Paracomesoma dubium* (6.29%), *Metalinhomoeus torosus* (4.4%), and *Steineria pilosa* (3.77%).

Significant differences characterized comparisons of controls and treatments with ciprofloxacin, as given in Table 2, principally resulted from abundance changes for the dominant taxa. The difference between structure of the control assemblage and that exposed to D1 was caused by the numerical increase in the proportion of *Paramonohystera pilosa*, and decrease of those of *Odontophora villoti*, *Paracomesoma dubium*, *Terschellengia longicaudata*, *Metoncholaimus pristiurus* and *Synonchiella edax*. The nematofauna collected from the treatment D2 was more distinct from controls. This resulted from an increase in the input of *Paramonohystera pilosa*, *Terschellengia longicaudata*, and *Synonchiella edax*, and the reduction of *Odontophora villoti*, *Paracomesoma dubium*, and *Metoncholaimus pristiurus*. The average dissimilarity between controls and treatment D3 resulted from the increased contribution of *Paramonohystera pilosa*, *Paracomesoma dubium*, *Terschellengia longicaudata*, and *Synonchiella edax*. Lastly, the difference between the control replicates and treatment with the highest concentration D4 was the consequence of the increased impact of *Paramonohystera pilosa*, *Metoncholaimus pristiurus*, and *Synonchiella edax*, and the reduction of *Odontophora villoti*, *Paracomesoma dubium*, and *Terschellengia longicaudata*.

A discernible difference was observed between ciprofloxacin-spiked and control substrates ($p < 0.05$) after multivariate analysis indicating that changes in abundance of the meiobenthic nematode taxa exposed to ciprofloxacin were related to the intensity of this fluoroquinolone antibiotic treatment. All contaminated microcosms were different from control replicates as shown in the MDS ordination (Fig. 2). The highest dose D4 of this pharmaceutical appeared as the most distinct from the control treatment in comparison with the others. Differences in the degree of sensitivity of the exposed taxa to the introduced antibiotic in the culture medium contribute to these modifications. Thus, the species *Odontophora villoti*, which was disadvantaged by all ciprofloxacin doses applied, may be classified as “ciprofloxacin-sensitive.” *Metoncholaimus pristiurus* was impacted by moderate concentrations and was increased in abundance at the highest dose D4; this species can thus be described as “opportunistic.” *Paramonohystera pilosa*, whose presence increased with increasing of the antibiotic concentration, appeared to be “ciprofloxacin-resistant” (Table 2).

4. Discussion

The negative response of Bizerte lagoon nematodes to experimental ciprofloxacin-enriched microcosms was in accord with ecotoxicological effects; practically all univariate indices considered discernibly decreased with ciprofloxacin treatment except for equitability (J'), which showed a significant enhance after the exposure to the intermediate concentrations applied, D2 and D3 (Fig. 1).

In our experiment, the impact of the antibiotic presence into the experimental enclosure on nematode species seems to occur through the easily uptake of surrounding ciprofloxacin from the sediment over their cuticles as well as the direct feeding of the part of the pollutant adsorbed on substrate particles (Boonsaner and Hawker, 2013; Li et al., 2012). Ciprofloxacin treatment caused a reduce in abundance and diversity, by deleting the sensitive taxa. Ciprofloxacin gathered on sediment particle surfaces shows a 3.5-fold increase in potency and has an affinity for adsorption (Halling-Sørensen et al., 2003; Tolls, 2001). Ciprofloxacin is reported to adsorb to sludge, sediments, and clay (Cardoza et al., 2005; Lindberg et al., 2005).

In addition, ciprofloxacin is not readily biodegradable (Kümmerer et al., 2000). It also strongly adsorbs to soil and sediments (Uslu et al., 2008), mostly by cation exchange (Vasudevan et al., 2009). Therefore, grain surfaces of these matrices may constitute a gathering areas for antibiotics (Rooklidge, 2004). In a previous study (Lillenberg et al., 2010), the concentration of ciprofloxacin was found to remain unchanged in these matrices during exposure to various doses (10, 50, 200 and 500 $\mu\text{g/g}$) during 28 days in the laboratory.

Ciprofloxacin is one of the fluoroquinolone antibiotics, and is very effective against various pathogenic bacteria including a wide range of gram-negative and a number of gram-positive organisms (Naora et al., 1999). The mode of action of ciprofloxacin involves inhibition of the bacterial enzymes, DNA gyrase and topoisomerase IV, which are required for replication and transcription in prokaryotic cells (Fisher et al., 1989; Hooper et al., 1987; Robinson et al., 2005). Quinolone antibiotics interact differently with the eukaryotic enzyme topoisomerase II, primarily because of differences in DNA structure, therefore the potential of genotoxic effects in eukaryotes is considerably lower compared to those in prokaryotic organisms (Toolaram et al., 2016). Several studies have shown that ciprofloxacin can modify the microbial community structure (i.e. abundance and diversity) in water, sediments, and soil (Cui et al., 2014; Girardi et al., 2011; Gonzalez-Martinez et al., 2014) from concentrations of 200 $\mu\text{g/L}$ and 0.1 $\mu\text{g/kg}$ (Näslund et al., 2008).

In our work, the density of marine free-living nematodes was significantly decreased with all tested doses, suggesting that this antibiotic by its antibacterial action, eliminated the bacteria that form the main food source of certain nematological trophic groups. Many studies have shown that the presence of antibiotics reduces microbial biodiversity. Moreover, they can influence the growth and enzyme activities of bacterial communities and ultimately ecological functions such as biomass production and nutrient transformation, leading to loss of functional stability (Pallecchi et al., 2008; Pauwels and Verstraete, 2006).

The taxonomic composition of aquatic bacterial communities was found to be changed significantly after exposure to 1 $\mu\text{g/L}$ of ciprofloxacin (Kraupner et al., 2018). In synthetic wastewater, ciprofloxacin reduced nitrification, denitrification, and phosphorus uptake at concentrations of 200–350 $\mu\text{g/L}$. This was accomplished

Table 2

Species responsible for differences between control and treated microcosms based on similarity percentages (SIMPER) analysis of square-root transformed data.

D1	D2	D3	D4
<i>Odontophora villoti</i> (–)	<i>Odontophora villoti</i> (–)	<i>Odontophora villoti</i> (–)	<i>Odontophora villoti</i> (–)
<i>Paramonohystera pilosa</i> (+)	<i>Paramonohystera pilosa</i> (+)	<i>Paramonohystera pilosa</i> (+)	<i>Paramonohystera pilosa</i> (+)
<i>Paracomesoma dubium</i> (–)	<i>Paracomesoma dubium</i> (–)	<i>Paracomesoma dubium</i> (+)	<i>Paracomesoma dubium</i> (–)
<i>Terschellengia longicaudata</i> (–)	<i>Terschellengia longicaudata</i> (+)	<i>Terschellengia longicaudata</i> (+)	<i>Terschellengia longicaudata</i> (–)
<i>Metoncholaimus pristiurus</i> (–)	<i>Metoncholaimus pristiurus</i> (–)	<i>Metoncholaimus pristiurus</i> (–)	<i>Metoncholaimus pristiurus</i> (+)
<i>Synonchiella edax</i> (–)	<i>Synonchiella edax</i> (+)	<i>Synonchiella edax</i> (+)	<i>Synonchiella edax</i> (+)

+ More abundant, – less abundant species. Species accounting for ~50% of the overall dissimilarity between treatment groups are ranked in the order of importance with respect to their contribution to this dissimilarity.

by a reduction in ammonium oxidizing bacteria, denitrifying bacteria, and polyphosphate-accumulating organism (Gonzalez-Martinez et al., 2014; Yi et al., 2017). In a wetland mesocosm (planted with *Phragmites australis* seeded with activated sludge from a WWTP) exposed to 2000 µg/L of ciprofloxacin (Weber et al., 2011), a temporary decrease in the activity and overall catabolic capabilities was observed in the bacterial communities along with decreased overall diversity of bacterial operational taxonomic units.

In aquatic organisms, principally in fish, scientific research has shown that ciprofloxacin (1000 µg/L) exerts adverse effects such as significant alteration in body weight and length at the early life stage of *Cyprinus carpio* (Zivna et al., 2016). Further, a greater hatching rate was observed at all concentrations (1–3000 µg/L), with reduced development in some larval stages at 1–500 µg/L, and accelerated development at 1000–3000 µg/L (Zivna et al., 2016). In zebrafish (*Danio rerio*), no effects on growth were observed at concentrations up to 3000 µg/L (Plhalova et al., 2014). Furthermore, dispersed results were obtained for some oxidative stress markers and enzyme activity in fish, suggesting that these effects were not always dose-response related (Zivna et al., 2016; Plhalova et al., 2014).

In invertebrates, ciprofloxacin showed no effects on the growth of two species *Gammarus* spp. and *Lepidostoma liba* (macroinvertebrates), upon exposure to 0.9 and 90 µg/L in experimental microcosms, respectively, for 45 days (Maul et al., 2006). In another study investigating the effects on Amphibia (*Rhinella arenarum*) exposed to 1, 10, 100 and 1000 µg/L for 4 days, ciprofloxacin reduced the larval length at 10 µg/L. A significant development inhibition greater than 10% was observed for the concentrations of 100 and 1000 µg/L, and additionally, GST levels were increased at 1000 µg/L (Peltzer et al., 2017). For meiofauna, marine nematodes have especially shown that the beta-lactam antibiotic, penicillin G, significantly decreases the univariate index including species number, Shannon index, Margalef's richness and Pielou's equitability after experimental treatment during 30 days (Nasri et al., 2015). Ciprofloxacin also restructured the trophic diversity of free-living nematodes in marine sediments experimentally enriched with increasing doses of the antibiotic (Nasri et al., 2014, 2016). These findings are in accord with our work.

Our study supported that ciprofloxacin has a negative effect on Mediterranean natural communities, specifically on the taxonomic diversity on meiobenthic nematofauna. These invertebrates showed clearly a significant response in function of the intensity of the antibiotic added. These results support the use of these particular organisms as bioindicative models toward antibiotics in biomonitoring programs of aquatic ecosystems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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