Ecological Indicators 120 (2021) 106868

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Toxic effects of phenanthrene intensify with an increase of temperature for the populations of a free-living nematode

Leticia Pereira Pontes[a], Anna-Maria Vafeiadou[b], Flavia Juliana Lobato de França[a], Raianne Amorim Cavalcante[a], Débora Alissandra de Araújo França[a], Clara Moura Brito[a], Romulo Nepomuceno Alves[a], Paulo Sérgio Martins de Carvalho[a], Giovanni Amadeu Paiva dos Santos[a,*]

[a] Universidade Federal de Pernambuco, Campus Recife, Centro de Biociências, Departamento de Zoologia, Av. Professor Moraes Rego, s/n. 50670-901, Recife, Pernambuco, Brazil

[b] Ghent University, Department of Biology, Marine Biology Research Group, Krijgslaan 281/S8, 9000 Ghent, Belgium

ARTICLE INFO

Keywords:
Diplolaimelloides delyi
Ecotoxicology
Global warming
Synergistic effect
Climate change
Bioindicators

ABSTRACT

Phenanthrene is one of the most common Polycyclic Aromatic Hydrocarbons (PAHs) in the marine environment. It has high lipoafinity and environmental persistence and tends to accumulate in benthic ecosystems. Exposure to phenanthrene can have severe impacts on a wide range of marine organisms, from nematodes to fish. These effects can be exacerbated with concurrent warming associated with climate change. In this study we investigated the response of free-living nematode populations of the species *Diplolaimelloides delyi* following exposure to different phenanthrene concentrations under normal and increased temperature conditions (from 25 °C up to 35 °C). Phenanthrene was toxic to *D. delyi*, causing a decrease in population growth (at concentrations ≥ 1 μg ml^{-1}) and negatively affecting their development times and reproduction (at concentrations ≥ 2.5 μg ml^{-1}). The observed effects intensified with increasing temperature, leading to further reduced development and population growth rate, arrested reproduction, and even mortality in 100% of the populations exposed to phenanthrene concentrations over 5 μg ml^{-1} at the highest temperature used (30 °C). Thermal-induced toxicity effects on marine populations can be significant, and current climate change and warming may have substantial implications for marine food webs and ecosystem functioning.

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a group of pollutants widely spread in the oceans (Cerniglia, 1993), even in remote environments such as the Antarctic or the Arctic Ocean and the deep sea (Dong et al., 2015; Stark et al., 2017). PAHs can be released in aquatic environments in various ways, including industrial discharges, accidental spills, gas and oil exploration, shipwrecks and stranded ships (Baguley et al., 2015; Ou et al., 2004; Stark et al., 2017). The continuous release of these toxic compounds, their lipoafinity, and their persistence and accumulation in marine ecosystems (Stringer et al., 2012), have caused increased environmental concern, particularly since PAHs are directly related to carcinogenicity (Cerniglia, 1993) and mutagenicity (Kennish, 1992). Research has shown that PAHs negatively impact marine organisms by causing physiological and metabolic changes, affecting their development, abundance and biomass, and ultimately their survival (Alves et al., 2017; Engraff et al., 2011; Louati et al., 2014; Nahrgang et al., 2013; Ren, 2002). Exposure to phenanthrene, in particular, has been related to behavioral effects, such as inability to coordinate muscle movements, locomotion and slow respiration in amphipods (Gauthier et al., 2016), reduced reproduction in copepods (Lotufo, 1997) and even mortality in copepods and polychaetes (Louati et al., 2014). The bioaccumulation of PAHs may also disrupt the marine environment on an ecosystem level (Haegerbaeumer et al., 2018), with effects being exacerbated when in combination with other environmental impacts associated with global change, such as warming (Macdonald et al., 2005; Noyes et al., 2009; Schiedek et al., 2007).

The continuously observed rise in temperatures is negatively impacting marine ecosystems worldwide (IPCC, 2014; Bindoff et al., 2019). Sea surface temperatures are estimated to rise from 1 °C to 4 °C by the end of the 21st century (Collins, 2013; IPCC, 2014; Bindoff et al.,

* Corresponding author.
E-mail address: giopaiva@hotmail.com (G.A.P. dos Santos).

https://doi.org/10.1016/j.ecolind.2020.106868
Received 8 April 2020; Received in revised form 11 August 2020; Accepted 18 August 2020

2019). Intertidal areas in particular are expected to be severely impacted, since these environments are also subjected to increased air temperature as well as daily temperature fluctuations (Helmuth et al., 2002). Temperature rise, along with other associated climatic events (i.e. ocean pH reduction, pollution increase, reduction in oxygen content, etc.) linked with climate change, can negatively affect species ecological niches, interspecific interactions and fitness (De Meester et al., 2015; Vafeiadou et al., 2018a). These changes lead to major disturbances in marine communities and may further impact the functioning of marine ecosystems (Danovaro et al., 2001). Although the accumulation of PAHs in the environment occurs concurrently with temperature increase, research on their synergistic effects is very limited, and usually focuses on specific organisms, such as fish (Laetz et al., 2014; Zebral et al., 2019). Studying the synergistic effects of PAHs and temperature increase on a range of different marine organisms is necessary to better understand and predict the potential disruption of marine populations and communities in future climate change situations (Nadal et al., 2015).

Marine free-living nematodes are meiofaunal organisms which occur in vast abundances in benthic ecosystems (Giere, 2009; Schratzberger and Ingels, 2018). Owing to their limited motility, their habitat (interstitial environment) and their semi-permeable cuticle, they are in direct contact with particles that are dissolved in water, such as contaminants (Giere, 2009). Nematodes can be easily cultivated in the laboratory (Moens et al., 2013) and are therefore ideal for studying response to changes in ecological conditions and the effects of contaminants experimentally. Marine free-living nematodes have already been used as model organisms in ecotoxicology (e.g. Gutiérrez et al., 2016; Martinez et al., 2018; Monteiro et al., 2014), population genetics (de Oliveira et al., 2017) and in studies testing the effects of temperature increase (e.g. Moens and Vincx, 2000a,b; Vafeiadou et al., 2018b, 2018a, Vafeiadou and Moens, 2021, this issue). In addition, they are very useful indicators of change in marine ecosystems, acting as sentinels to shifts in environmental parameters (Balsamo et al., 2012; Bongers and Ferris, 1999; Semprucci and Balsamo, 2012).

Only a few studies have examined the effects of phenanthrene on meiofauna and nematode communities in the marine environment (Louati et al., 2014, 2015) as well as on terrestrial free-living nematode species, such as *Caenorhabditis elegans* (Spann et al., 2015). However, none of these studies have investigated the effect of phenanthrene on specific species of marine free-living nematodes, nor have they assessed the potential of synergistic effects with warming. Based on existing knowledge for the nematode genera *Diplolaimelloides* tolerating temperature variations from 10 to 30 °C, with optimum developmental conditions around 20 °C (Moens and Vincx, 2000a,b), we degined our experiment testing the effects of phenanthrene in combination with temperature increase. Here we investigated the effects of phenanthrene and/or warming on population parameters of the marine nematode species *Diplolaimelloides delyi* Andrassy, 1958, using six different phenantrene concentrations (based on environmental concentrations, and effect and threshold levels), five different temperature regimes (average *in-situ* temperature and higher), and twelve combination treatments (six different phenanthrene concentrations crossed with two temperature regimes and controls) in an experimental setup. Based on the premises that PAHs have toxic effects on several marine benthic organisms (Louati et al., 2014) and on evidence of additive effects of other contaminants with warming (Schiedek et al., 2007), we tested the following hypotheses: (1) the population growth and development of *D. delyi* will decrease with increasing phenanthrene concentrations; (2) the population growth and development of *D. delyi* will decrease with increasing temperature; (3) the effect of phenanthrene will be stronger with increasing temperature. These hypotheses allow us to investigate whether marine nematodes are useful as ecological indicators to assess the synergistic effects of a contaminant and warming, especially in the context of climate change.

2. Materials and methods

2.1. Model species

Species of the genus *Diplolaimelloides* are generalists and, like other representatives of the family Monhysteridae, feed mainly on bacteria, but small protists can also be part of their diet (Moens and Vincx, 1997). Our test species was *Diplolaimelloides delyi*, which occurs in high abundances in nutrient-rich environments (colonizer-persistent scale cp2; Bongers and Bongers, 1998; Bongers and Ferris, 1999; De Ward and Russo, 2009) and, like other *Diplolaimelloides* species, is known as bacterivorous with a short life-cycle and fast reproduction rate, as well as high numbers of offspring. *D. delyi* has so far been used in studies assessing the toxicity of organophosphate pesticides (Newell et al., 1981) and investigating nematode phylogeny (Van Gaever et al., 2009), while species of the genus *Diplolaimelloides* have been used as model species in experimental studies investigating the effects of temperature and salinity (Moens and Vincx, 2000ab; Vafeiadou et al., 2018a) and food concentrations (Dos Santos et al., 2008).

2.2. Sampling

Sampling was carried out in 2017, at Cupe beach, near the city of Ipojuca –PE, Brazil (8° 27′ 29.4″S 34° 59′ 3.2″W), which is characterized by sandy sediments. The average daily interstitial temperature at the sampling site ranges between 25 °C − 30 °C. Sediments were collected at the low intertidal zone, during low tide, by scooping up the superficial (0–2 cm) sediment layer. The sediments were immediately taken to the laboratory where they were used to establish isolation cultures of the test species, following the protocol by Moens and Vincx (1998). All the individuals used in the experiments originated from these cultures.

2.3. Experimental design

Three independent microcosm experiments were set up: (1) nematodes were exposed to six different concentrations of phenanthrene and two different controls at a constant temperature to investigate the effect of the contaminant alone; (2) nematodes were incubated at five different temperature regimes to investigate a potential temperature effect; and (3) nematodes were exposed to six different concentrations of phenanthrene and two different contols at two constant temperatures (30 °C and 32 °C), to investigate a potential interaction effect of phenanthrene and temperature.

All experiments were conducted in microcosms of 5-cm diameter Petri dishes filled with 5 ml of sterile bacto-agar diluted in distilled water (6 g l^{-1}), each with a salinity of 25 (Instant Ocean was added) (Moens and Vincx, 1998). The agar was previously enriched with 100 µl l^{-1} of cholesterol, because nematodes cannot synthesize steroids when their only food source is bacteria (Vanfleteren, 1980).

2.4. Experimental set-up

2.4.1. Exposure of Diplolaimelloides delyi to different concentrations of phenanthrene

Different concentrations of phenanthrene were created from a stock solution (200 ppm) of phenanthrene and acetone mixed with a bacto-agar medium as described in 2.3. Phenanthrene (98% pure, Sigma Aldrich) was diluted with 99% pure acetone resulting in the following experimental solutions: Control (C; no contaminant), Solvent control (CS; 50 µl acetone), 0.1 µg ml^{-1}, 1 µg ml^{-1}, 2.5 µg ml^{-1}, 5 µg ml^{-1}, 10 µg ml^{-1} and 20 µg ml^{-1} phenanthrene. This concentration gradient was based on phenanthrene analyses in sediment and water (Macdonald et al., 2000; McCready et al., 2006), as well as on the range of Probable Effect Levels (PEL) and Threshold Effect Levels (TEL) for marine biota (Macdonald, 1994). A pilot experiment showed that the concentration of acetone did not significantly affect the population of *D.*

delyi (authors' unpublished data). The solutions were mixed with agar and four replicate plates were prepared for each experimental treatment; in total 32 plates were prepared. Then, 10 male and 10 female nematode individuals were transferred to a drop (100 µl) of sterile seawater on the agar surface of each plate. Additional food was added to each microcosm in the beginning of the experiment and after 15 days (50 µl of thawed *Escherichia coli*, 3×10^{10} cells ml^{-1}). Counts of males, females, juveniles and eggs were performed under a stereomicroscope (customized Stemi-305, from Zeiss) every 2 days for a period of 30 days.

2.4.2. Exposure of Diplolaimelloides delyi to different temperature regimes

Microcosms for this experiment were incubated in the dark, in temperature-regulated incubators at five different constant temperatures: 25 °C, 28 °C, 30 °C, 32 °C and 35 °C. The lowest temperature treatment was based on the average sediment temperature at the sampling site (25 °C), while 30 °C comprises peak temperature recordings on the warmest days. The temperature treatments of 32 °C and 35 °C were chosen to simulate global warming scenarios over the next 50 and 100 years (IPCC, 2014); we used the average sediment temperature of 25 °C as control for this experiment. Four replicate microcosms corresponded to each experimental treatment; in total 20 plates were prepared. Ten male and 10 female nematode individuals were transferred to a drop (100 µl) of sterile seawater on the agar surface of each plate. Additional food was added to each microcosm in the beginning of the experiment and after 15 days (50 µl of thawed *E. coli*, $3 \times\times 10^{10}$ cells ml^{-1}). Counts of males, females, juveniles and eggs were performed every 2 days for a period of 30 days.

2.4.3. Exposure of Diplolaimelloides delyi to different phenanthrene concentrations at increased temperature

Concentrations of phenanthrene in this experiment were the same as in the phenanthrene-only experiment (see Section 2.4.1), with eight treatments in total (including control and solvent control; C and CS respectively), and crossed with three temperatures: 25 °C (Control, as in phenanthrene-only experiment), 30 °C (maximum sediment temperature at the sampling site), and 32 °C (IPCC 50-year warming prediction). The temperature of 35 °C was not used in this phenanthrene-combination experiment because a preliminary experiment resulted in 100% mortality of nematodes at 35 °C. Four replicates were prepared per treatment, resulting in 32 plates for each temperature. Ten male and 10 female nematode individuals were transferred to a drop (100 µl) of sterile seawater on the agar surface of each plate. Additional food was added to each microcosm in the beginning of the experiment and after 15 days (50 µl of thawed *E. coli*, 3×10^{10} cells ml^{-1}). The plates were incubated in the dark at controlled constant temperature of 30 °C and 32 °C. Counts of males, females, juveniles and eggs were performed every 2 days for a period of 30 days.

2.5. Data analysis

The following population parameters were calculated: pre-embryonic development time (PREDT), post-embryonic development time (PEDT), total development time (TDT), sex ratio, daily reproductive output (DR) and total reproductive output (TR). The latter two were based on the total number of eggs laid daily and the cumulative number of eggs laid during the total experiment duration, respectively (adapted from Moens and Vincx, 2000a). The population parameters adult and juvenile population growth, and the number of eggs over time were analyzed using two Sigmaplot V.12.5 routines: (i) exponential growth (population) curve models ($f = a/(1 + \exp(-(x-x0)/b))$) were used to create the best fitted population curves per treatment and were tested for overall best fit with $R > 0.9$ and (ii) the area under the curve (AUC) was calculated per treatment, with data normality being tested with the Shapiro-Wilk test. Population parameters were analyzed using a non-parametric Permutational Multivariate Analysis of Variance (PERMANOVA) in PRIMER v6. The similarity matrix was constructed using the Euclidean distance with 9999 permutations to assess whether there were significant differences in the population parameters measured in each experiment separately (Phenanthrene, Temperature and Phenanthrene with increased temperature). Pairwise comparisons between treatments in each experiment were performed when significant results were obtained ($p < 0.05$). The PERMDISP test was performed in each experiment to test the homogeneity of dispersions.

3. Results

3.1. Population growth, development and reproduction of Diplolaimelloides delyi exposed to different phenanthrene concentrations at control temperature (25 °C)

3.1.1. Population growth of D. delyi exposed to different phenanthrene concentrations at control temperature (25 °C)

Significant changes in the adult population growth of *D. delyi* were observed in treatments with different phenanthrene concentrations ($df = 7$; Pseudo-F = 220; $p = 0.0001$; PERMDISP: $p = 0.06$). The highest adult abundances were observed in the C and CS treatments, and in the treatment with a phenanthrene concentration of 0.1 µg ml^{-1} ($p > 0.05$); which were significantly higher than in other treatments ($p < 0.05$; Fig. 1A). Adult growth was not observed in the treatments with the two highest phenanthrene concentrations (10 and 20 µg ml^{-1}).

A significant effect of phenanthrene on the juvenile population growth was observed ($df = 7$; Pseudo-F = 500; $p = 0.0001$; PERMDISP: $p = 0.17$), with highest juvenile abundances at 0.1 µg ml^{-1} phenanthrene. At 5 µg ml^{-1} phenanthrene, we observed the fastest juvenile population growth but differences with the C, CS, 0.1 µg ml^{-1} phenanthrene treatment were not significant ($p > 0.05$; Fig. 1B). Juvenile population growth at concentrations of 2.5 µg ml^{-1} and 10 µg ml^{-1} differed significantly from all other treatments ($p < 0.05$). Juveniles in the treatment with 10 µg ml^{-1} phenanthrene did not mature, and eventually died, while in the 20 µg ml^{-1} treatment, eggs did not hatch and thus, juveniles were not observed.

Differences in the number of eggs were also observed among the different phenanthrene treatments ($df = 7$; Pseudo-F = 451; $p = 0.0001$; PERMDISP: $p = 0.11$), with the highest number of eggs in the controls C and CS, and in the treatment with the lowest phenanthrene concentration (0.1 µg ml^{-1}; $p > 0.05$). These treatments differed significantly from all other treatments ($p < 0.05$; Fig. 1C). In the 10 µg ml^{-1} phenanthrene treatment, a slight increase in the number of eggs was observed, but no eggs were observed after 11 days, whereas in the treatment with a concentration of 20 µg ml^{-1}, reproduction was not successful.

3.1.2. Development time of D. delyi exposed to different concentrations of phenanthrene at control temperature (25 °C)

The total development time (TDT) differed significantly between treatments ($df = 5$; Pseudo-F = 6.37; $p = 0.002$; PERMDISP: $p = 0.4$). The highest TDT was observed in the 2.5 and 5 µg ml^{-1} phenanthrene treatments (17.4 ± 0.51 days and 18.01 ± 0.2 days, respectively), which were significantly different from all other treatments ($p < 0.05$; Table 1). The post-embryonic development time (PEDT) varied significantly between treatments ($df = 5$; Pseudo-F = 3.73; $p = 0.01$; PERMDISP: $p = 0.7$), with the longest PEDT (12.52 ± 0.22 days) being observed at 5 µg ml^{-1} phenanthrene ($p < 0.05$). The pre-embryonic development time (PREDT) was not significantly affected by phenanthrene exposure, independently of the concentration level ($df = 6$; Pseudo-F = 2.23; $p = 0.08$; PERMDISP: $p = 0.06$; Table 1).

3.1.3. Reproductive output and sex ratio of D. delyi populations exposed to different concentrations of phenanthrene at room temperature (25 °C)

Daily (DR) and total reproductive output (TR) were significantly affected by phenanthrene ($df = 6$; Pseudo-F = 442.9; $p = 0.0001$;

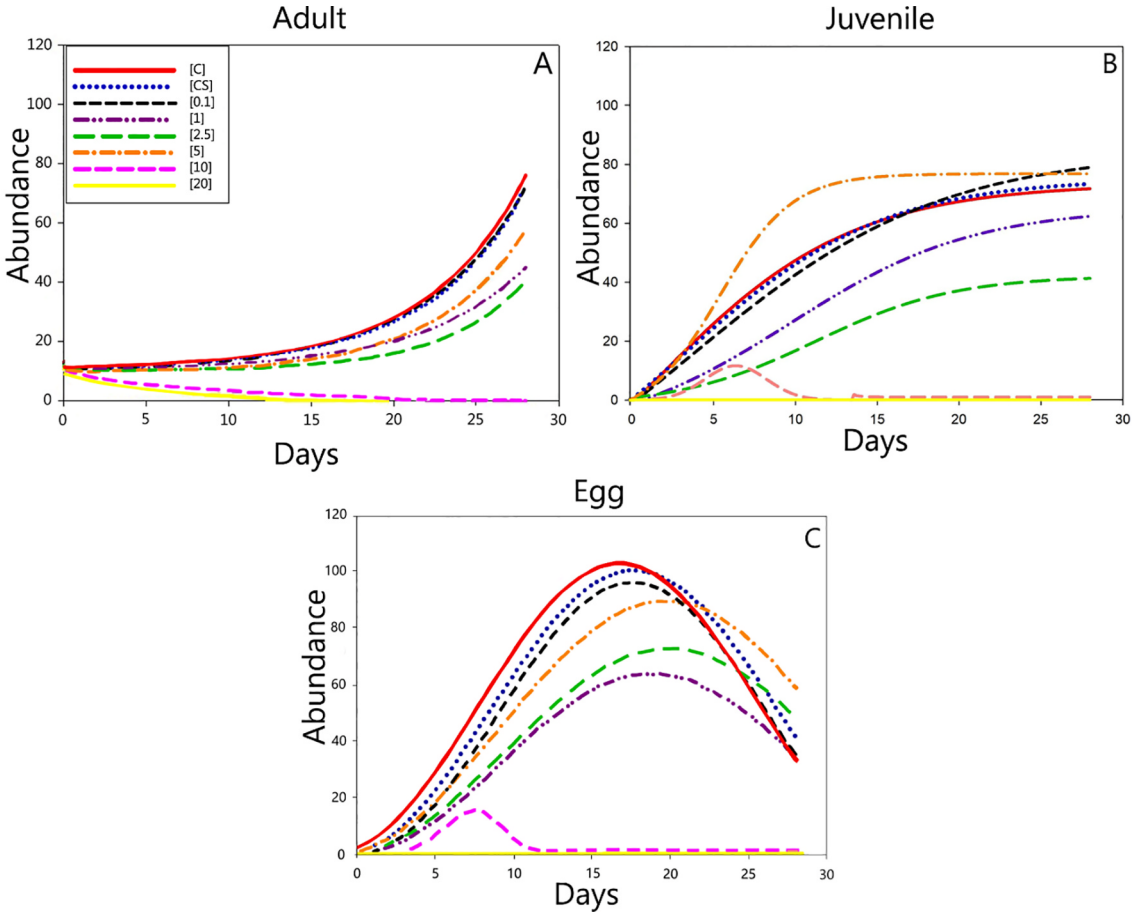

Fig. 1. Adjusted model curves of the variation trend of the mean abundance in *Diplolaimelloides delyi* adult (A) and juvenile individuals (B) and egg numbers (C) over time (days) incubated at different treatments tested: control (C), solvent control (CS) and different phenanthrene concentrations (0.1, 1, 2.5, 5, 10 and 20 μg ml^{-1}) at 25 °C. Starting points of curves do not overlap for better visualization of the model.

Table 1
Mean and standard error of Pre-embryonic Development Time (PREDT), Post-embryonic Development Time (PEDT) and Total Development Time (TDT) of *Diplolaimelloides delyi* in days, at different phenanthrene concentrations at 25 °C. The (*) refers to parameters that differed significantly from the control ($p < 0.05$).

Concentrations	PREDT	PEDT	TDT
Control	3.23 ± 0.21	10.19 ± 0.33	15.99 ± 0.44
CS	3.47 ± 0.1	9.48 ± 0.68	15.21 ± 0.47
0.1 μg ml^{-1}	3.02 ± 0.28	9.9 ± 0.21	15.57 ± 0.23
1 μg ml^{-1}	2.76 ± 0.32	9.39 ± 0.42	15.72 ± 0.21
2.5 μg ml^{-1}	2.68 ± 0.54	10.98 ± 0.54	17.40 ± 0.51*
5 μg ml^{-1}	3.16 ± 0.19	12.52 ± 0.22*	18.01 ± 0.20*
10 μg ml^{-1}	3.20 ± 0.22	–	–
20 μg ml^{-1}	–	–	–

PERMDISP: $p = 0.16$). Reproductive output in the treatments with 2.5 and 10 μg ml^{-1} phenanthrene differed significantly between each other and from all other treatments ($p < 0.05$, for both DR and TR), with the lowest egg deposition being recorded at 10 μg ml^{-1} phenanthrene (Table 2).

A slight female dominance was observed in all treatments, and despite the fact that the female:total adult ratio decreased with increasing phenanthrene concentrations, sex ratio differences between treatments were not significant (df = 6; Pseudo-F = 0.79; $p = 0.56$; PERMDISP: $p = 0.3$; ESM:Table 1).

Table 2
Mean and Standard Error of total and daily reproductive output of *Diplolaimelloides delyi* in treatments with different phenanthrene concentrations at 25 °C. The upper case letters (^{a,b} and ^{c}) refer to groups of the parameters that differed significantly among each other ($p < 0.05$). The (–) refers to the absence of eggs for the present analysis.

Treatment	Total reproductive output (total number of eggs laid during all experimental days)	Daily reproductive output (total number of eggs/days of experiment)
C	548 ± 24.48^{c}	19.57 ± 0.87^{c}
CS	513 ± 33.17^{c}	18,32 ± 1.18^{c}
0.1 μg ml^{-1}	591 ± 29.32^{c}	21.11 ± 1.04^{c}
1 μg ml^{-1}	515 ± 12.19^{c}	18.39 ± 0.43^{c}
2.5 μg ml^{-1}	426 ± 8.61^{b}	15.22 ± 0.30^{b}
5 μg ml^{-1}	537 ± 14.67^{c}	19.18 ± 0.52^{c}
10 μg ml^{-1}	11 ± 2.01^{a}	0.4 ± 0.07^{a}
20 μg ml^{-1}	–	–

3.2. Population growth, development and reproduction of Diplolaimelloides delyi exposed to different temperatures

3.2.1. Population growth of D. delyi exposed to different temperatures

Adult population growth significantly decreased with temperature increase (df = 4; Pseudo-F = 24.35; $p = 0.0001$; PERMDISP: $p = 0.063$). The highest adult abundances were recorded in the two lowest temperature treatments (25 °C and 28 °C; $p = 0.19$), and differed significantly from the adult abundances in the other temperature treatments (30 °C, 32 °C and 35 °C; $p < 0.05$). No F1-generation adults matured at 35 °C (Fig. 2A).

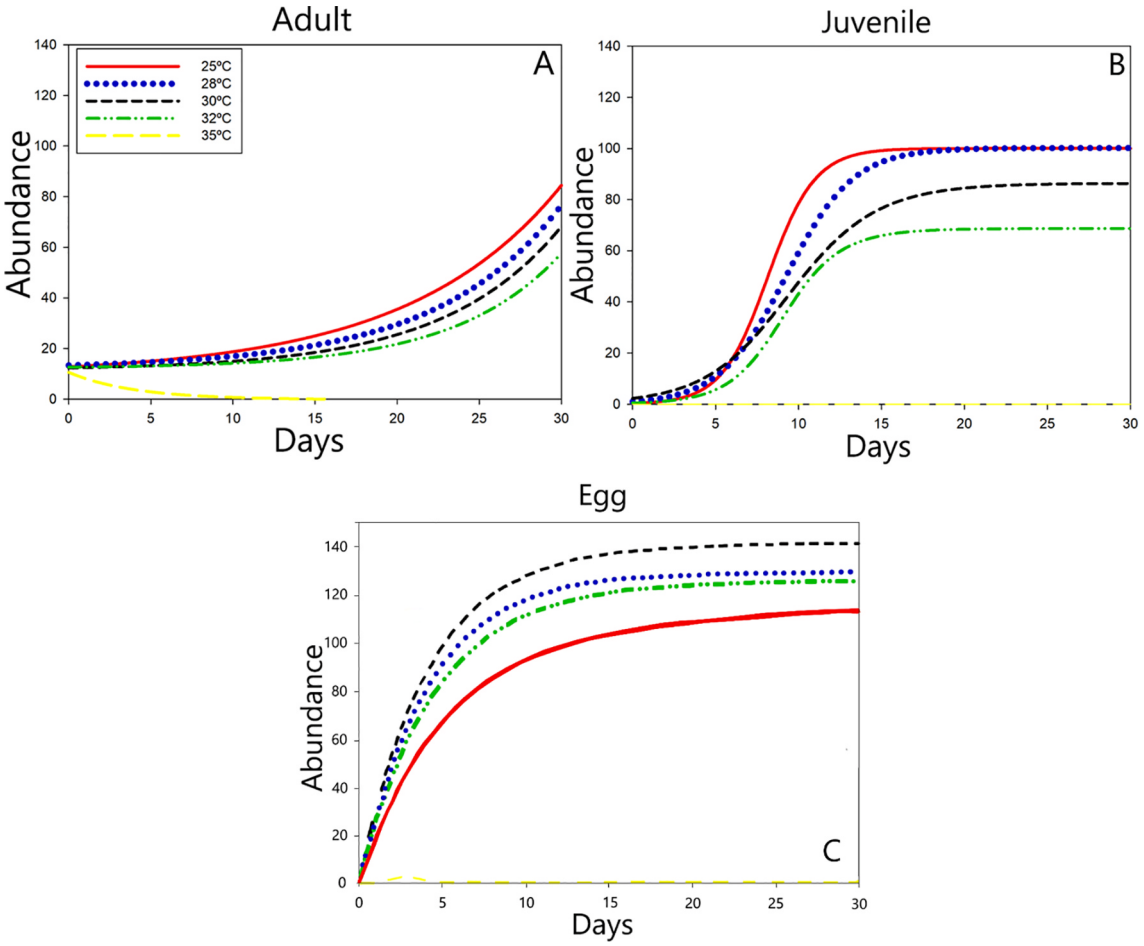

Fig. 2. Adjusted model curves of the variation trend of the mean abundance in *Diplolaimelloides delyi* adult (A) and juvenile individuals (B) and egg numbers (C) over time (days) incubated at different temperatures (25 °C, 28 °C, 30 °C, 32 °C, 35 °C). Starting points of curves do not overlap for better visualization of the model.

Juvenile population growth was similarly affected by temperature (df = 4; Pseudo-F = 2909.9; p = 0.0001; PERMDISP: p = 0.18), with significantly higher juvenile abundances being observed at a temperature of 25° and 28 °C (which did not differ, p > 0.05) compared to the other temperature treatments (p < 0.05). Juvenile population growth reached a plateau after 15 days in all temperature treatments, except for 35 °C. No juvenile development was observed in the 35 °C treatment (Fig. 2B).

The total number of eggs differed at different temperatures (df = 4; Pseudo-F = 357.17; p = 0.0001; PERMDISP: p = 0.18), with the highest number at 30 °C (Fig. 2C). Number of eggs differed significantly in the 35 °C and 25 °C treatments, compared to all other treatments (p < 0.05) (Fig. 2C).

3.2.2. Development time of *D. delyi* exposed to different temperatures

Pre-embryonic development time (PREDT) and total development time (TDT) decreased with increasing temperature (df = 3 Pseudo-F = 49.8; p = 0.013; PERMDISP: p = 0.12 and df = 3; Pseudo-F = 4.23; p = 0.023; PERMDISP: p = 0.53, respectively; Table 3). The shortest PREDT and TDT were observed at 32 °C (2.29 ± 0.29 days and 13.55 ± 0.42 days, respectively) and both differed significantly from the respective development times in all other temperature treatments (p < 0.05). Post-embryonic development time (PEDT) did not differ significantly among different temperature treatments (df = 3; Pseudo-F = 0.5; p = 0.63; PERMDISP: 0.5).

Table 3
Mean and Standard Error of Pre-embryonic Development Time (PREDT), Post-embryonic Development Time (PEDT) and Total Development Time (TDT) of *Diplolaimelloides delyi* in days, at different temperatures. The (*) refers to the parameters that differed significantly from the control (25 °C) (p < 0.05).

Temperature	PREDT	PEDT	TDT
25 °C	3.67 ± 0.28	10.66 ± 0.19	15.33 ± 0.20
28 °C	3.74 ± 0.30	10.55 ± 0.30	15.29 ± 0.49
30 °C	2.5 ± 0.39	10.22 ± 0.12	14.72 ± 0.31
32 °C	2.29 ± 0.29*	9.72 ± 0.44	13.55 ± 0.42*
35 °C	–	–	–

3.2.3. Reproductive output and sex ratio of *D. delyi* populations exposed to different temperatures

Both daily reproductive output (DR) and total reproductive output (TR) significantly increased with temperature increase from 25 °C to 32 °C (df = 4; Pseudo-F = 119.66; p = 0.0002; PERMDISP: p = 0.16), except for the 35 °C treatment. The lowest reproductive output was observed in the population exposed to 35 °C, differing significantly from the control and all other treatments (p < 0.05). The second lowest reproductive output was observed in the control (25 °C), and also differed significantly from all other temperature treatments (p < 0.05; Table 4).

Sex ratio was not significantly affected by temperature (df = 4 Pseudo-F = 3.41; p = 0.05; PERMDISP: p = 0.3), and a female dominance was observed in all treatments (See ESM:Table 2).

Table 4
Mean and Standard Error of total and daily reproductive output of *Diplolaimelloides delyi* at different temperatures (25 °C, 28 °C, 30 °C, 32 °C, 35 °C). The upper case letters ([a,b and c]) refer to groups of the parameters that differed significantly among each other ($p < 0.05$).

Treatment	Total reproductive output (total number of eggs laid during all experimental days)	Daily reproductive output (total number of eggs/days of experiment)
25 °C	813 ± 15.57[b]	27.12 ± 1.18[b]
28 °C	1142 ± 36.61[c]	38.07 ± 1.22[c]
30 °C	1244 ± 148.82[c]	41.48 ± 4.96[c]
32 °C	1094 ± 98.17[c]	36.48 ± 3.27[c]
35 °C	4 ± 0.75[a]	0.15 ± 0.02[a]

3.3. Population growth, development and reproduction of Diplolaimelloides delyi exposed to different phenanthrene concentrations at increased temperature

3.3.1. Population growth of D. delyi exposed to different phenanthrene concentrations at increased temperature

Adult population growth was significantly affected by the interaction of phenanthrene and elevated temperature (df = 7; Pseudo-F = 489; p = 0.0001; PERMDISP: p = 0.316). The most severe effects were observed at 32 °C, which was proved lethal to *D. delyi* in combination with the exposure to phenanthrene. At 30 °C, the highest adult abundances were observed in the two control treatments (C and CS; p = 0.79), which differed significantly from all other treatments ($p < 0.05$). Adult development was not successful in treatments exposed to a phenanthrene concentration of 5 μg ml^{-1}, 10 μg ml^{-1} and 20 μg ml^{-1} (Fig. 3A).

Juvenile population growth was also significantly affected by exposure to phenanthrene at 30 °C (df = 7; Pseudo-F = 459; p = 0.0001; PERMDISP: p = 0.081). The highest juvenile abundances were observed in the two control treatments (C and CS; p = 0.54), compared to these in all other treatments ($p < 0.05$; Fig. 3B). Juvenile development was limited only to the first 10 days in the treatment with 5 μg ml^{-1} phenanthrene, whereas no juvenile development occurred at the highest phenanthrene concentrations (10 and 20 μg ml^{-1}) at this temperature (30 °C).

The total number of eggs was similarly impacted by exposure to phenanthrene at an increased temperature (df = 7; Pseudo-F = 97,44; p = 0.0001; PERMDISP: p = 0.06), the highest numbers being recorded in the control treatments (C and CS; p = 0.32), significantly higher compared to all other treatments ($p < 0.05$). The total number of eggs at low phenanthrene concentrations (0.1, 1 and 2.5 μg ml^{-1}) also differed from all other treatments ($p < 0.05$; Fig. 3C). A slight increase in the number of eggs was observed up to the 8th day at 5 μg ml^{-1} of phenanthrene. However, egg numbers declined towards the end of the experiment compared to the other phenanthrene concentrations ($p < 0.05$; Fig. 3C). No eggs were observed in the treatments with the two highest phenanthrene concentrations (10 and 20 μg ml^{-1}) at 30 °C.

3.3.2. Development time of D. delyi exposed to different phenanthrene concentrations at 30 °C

Total development time (TDT) increased significantly with

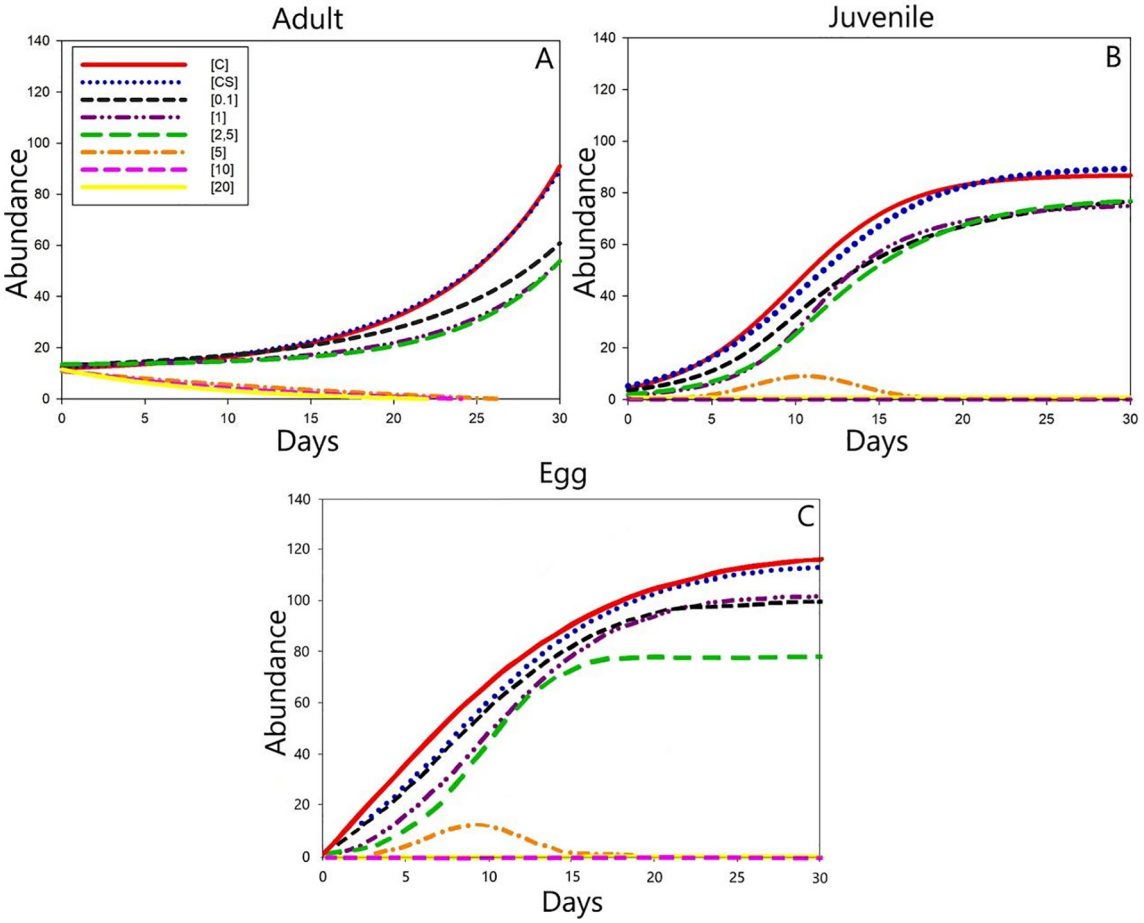

Fig. 3. Adjusted model curves of the variation trend of the mean abundance in *Diplolaimelloides delyi* adult (A) and juvenile individuals (B) and egg numbers (C) over time (days) incubated in different treatments tested: control (C), solvent control (CS) and different phenanthrene concentrations (0.1, 1, 2.5, 5, 10 and 20 μg ml^{-1}) at 30 °C. Starting points of curves do not overlap for better visualization of the model.

Table 5
Mean and standard error of Total Development Time (TDT), Pre-embryonic Development Time (PREDT) and Post-embryonic Development Time (PEDT) of *Diplolaimelloides delyi* in days, at different phenanthrene concentrations at 30 °C. The (*) refers to parameters that differed significantly from the control ($p < 0.05$).

Concentrations	PREDT	PEDT	TDT
Control	3.17 ± 0.53	10.26 ± 0.26	15.44 ± 0.66
CS	3.18 ± 0.54	10.40 ± 0.96	15.22 ± 0.51
0.1 μg ml^{-1}	2.86 ± 0.44	9.8 ± 0.35	15.67 ± 0.41
1 μg ml^{-1}	2.97 ± 0.17	9.21 ± 0.29	17.18 ± 0.34*
2.5 μg ml^{-1}	3.01 ± 0.45	9.47 ± 0.55	18.04 ± 0.31*
5 μg ml^{-1}	2.95 ± 0.10	–	–
10 μg ml^{-1}	–	–	–
20 μg ml^{-1}	–	–	–

increasing phenanthrene concentration at 30 °C (df = 4: Pseudo-F = 15.21; p = 0.0003; PERMDISP: p = 0.79; Table 5). The highest TDT was observed in the treatments with a concentration of 1 and 2.5 μg ml^{-1} (17.18 ± 0.34 days and 18.04 ± 0.31 days, respectively), and differed significantly from those at 0.1 μg ml^{-1} and in the controls (C and CS, $p < 0.05$). There were no significant differences in the pre-embryonic (PREDT) and post-embryonic (PEDT) development time between different phenanthrene concentrations at 30 °C (PREDT: df = 5; pseudo-F = 0.51; p = 0.76; PERMDISP: p = 0.23; and PEDT: df = 4; pseudo-F = 2.55; p = 0.085; PERMDISP: p = 0.72, respectively). PREDT data were not obtained at concentrations of 10 μg ml^{-1} and 20 μg ml^{-1}, due to the absence of eggs and juvenile growth. Similarly, PEDT and TDT were not measured at concentrations of 5, 10 and 20 μg ml^{-1}, because adult development was not observed in these treatments.

3.3.3. Reproductive output and sex ratio of D. delyi populations exposed to different phenanthrene concentrations at 30 °C

Daily (DR) and total reproductive output (TR) were significantly reduced with increasing phenanthrene exposure at 30 °C (df = 5; Pseudo-F = 1652.9; p = 0.0001; PERMDISP: p = 0.052). The lowest reproductive output was observed at 2.5 and 10 μg ml^{-1} phenanthrene ($p > 0.05$ for both DR and TR), and differed significantly from all other treatments ($p < 0.05$ for both DR and TR; Table 6).

A slight female dominance was observed in all treatments, and despite females:total adult ratios decreasing with increasing phenanthrene concentrations (See ESM:Table 3), sex ratio differences between treatments were not significant (df = 4; Pseudo-F = 1.38; p = 0.28; PERMDISP: p = 0.57).

Table 6
Mean and Standard Error of total and daily reproductive output of *Diplolaimelloides delyi* in treatments with different phenanthrene concentrations at 30 °C. The upper case letters (^{a,b} and ^{c}) refer to groups of the parameters that differed significantly among each other ($p < 0.05$). The (–) refers to the absence of eggs for the present analysis.

Treatment	Total reproductive output (total number of eggs laid during all experimental days)	Daily reproductive output (total number of eggs/days of experiment)
C	1070 ± 34.85^{c}	35.66 ± 1.16^{c}
CS	1016 ± 22.97^{c}	34.86 ± 0.76^{c}
0.1 μg ml^{-1}	911. 25 ± 11.35^{b}	30.37 ± 0.37^{b}
1 μg ml^{-1}	913 ± 4.5^{b}	30.43 ± 0.15^{b}
2.5 μg ml^{-1}	816 ± 12.48^{b}	27.2 ± 0.41^{b}
5 μg ml^{-1}	54.5 ± 3.77^{a}	1.81 ± 0.12^{a}
10 μg ml^{-1}	–	–
20 μg ml^{-1}	–	–

4. Discussion

The marine free-living nematode species *Diplolaimelloides delyi* is a useful and accessible model species for ecotoxicological research, owing to its easy cultivation and documented resistance to pollutants. The life-history and physiological responses of this species to phenanthrene exposure resembled responses of other *Diplolaimelloides* species exposed to heavy-metals (Bogaert et al., 1984; Bendoy et al., 2014) or to mixed crude-oil contaminants (Monteiro et al., 2018). Species of the genus *Diplolaimelloides* have also been used as model organisms in a range of experimental studies with focus on various environmental conditions (Dos Santos et al., 2008, 2009; Moens and Vincx, 2000a,b; Vafeiadou et al., 2018a), suggesting that members of this genus are efficient bioindicators for ecological research.

4.1. Effects of phenanthrene on Diplolaimelloides delyi populations at control temperature (25 °C)

Diplolaimelloides delyi was sensitive to phenanthrene exposure in our study, which resulted in significant reduction in population growth compared to the control treatments (no contaminant). Such reduction in population growth has also been reported for other meiofauna organisms, i.e. copepods (Evans and Nipper, 2007; Stringer et al., 2012), and for the model soil nematode species *Caenorhabditis elegans* (Sese et al., 2009). Although these studies had a shorter experimental incubation time (48 to 72 h), and used concentrations similar to the lower phenanthrene concentrations (from 0.06 to 2.4 μg ml^{-1}) used in our experiment, their results revealed a stronger phenanthrene effect than the effect reported here. *Diplolaimelloides delyi*, as a generalist nematode, has a slight resistance to phenanthrene compared to copepods (Evans and Nipper, 2007; Stringer et al., 2012), partly because of the permeability of the nematode cuticle that reduces the passive uptake of some contaminants (Moens et al., 2013). The observed increase in juvenile abundances of *D. delyi* at 5 μg ml^{-1} phenanthrene was unexpected. However, the increase in juvenile abundances was not followed by a respective increase in adult abundances, which may be explained by the significant delay of the post-embryonic development (juvenile development), and the non-maturation of juveniles afterwards. The significant increase in total development time (TDT) at a phenanthrene concentration of 2.5 and 5 μg ml^{-1} compared to the control, coinciding with slight increases in pre-embryonic (PREDT) and post-embryonic development time (PDT), respectively, implies an overall delay in organismal development from oviposition until adulthood.

The significant decrease in daily and total egg deposition at 2.5 and 10 μg ml^{-1} phenanthrene has also been reported for calanoid copepods exposed to lower phenanthrene concentrations (0.32 and 0.42 μg ml^{-1}) (Bellas and Thor, 2007). The decreased number of nematode eggs at these concentrations is probably related to the fact that phenanthrene, as a hydrophobic compound, tends to connect to the animal's lipophilic regions, including gonads (Spann et al., 2015), thus leading to contamination of the egg lipid reserve. A contamination of gonads and/or eggs likely leads to a reduction in the number of eggs laid and hatched. A decrease in egg deposition, in combination with reduced juvenile development, resulted in reduced population growth with increasing phenanthrene concentrations in our experiment.

Furthermore, phenanthrene accumulation in nematode lipophilic tissues without a lethal effect can lead to further bioaccumulation of the contaminant, not only in the subsequent nematode generations but also in other marine fauna, following consumption of nematodes up the food web into higher trophic levels (Louati et al., 2014, Watanabe et al., 2005). Further experiments measuring the bioaccumulation potential of *D. delyi*, and the transfer of phenanthrene to higher-level consumers of contaminated nematodes are necessary in order to investigate whether phenanthrene can cause a disruption of marine food webs.

Some types of PAHs, including phenanthrene, are known as

endocrine disruptors (Zhang et al., 2016), and often result in sex ratio modification. However, the phenanthrene concentrations used in our experiment did not affect significantly the sex ratio of the *D. delyi* populations, but a slight reduction in adult female proportions with exposure to increasing phenanthrene concentrations was observed. Further studies, examining different phenanthrene concentrations over longer exposure times and using many more nematode generations, are needed to assess whether nematode sex ratio can serve as an accurate indication of phenanthrene as an endocrine disruptor.

4.2. Exposure of Diplolaimelloides delyi to different temperatures

Temperature rise is one of the most important anthropogenic consequences of climate change worldwide, which, along with the intensity and frequency of extreme climatic events, will impact ecosystem structure and functions severely. Population fitness and life histories of ectothermic species, such as nematodes, are very likely to be highly impacted by the consequences of global change, particularly warming (Ruess et al., 1999). Studies have reported negative impacts of warming beyond species-specific thresholds on nematode growth, reproduction and population life-history and development for several free-living terrestrial, fresh-water (Majdi et al., 2019) and marine nematode species (Moens and Vincx, 2000a,b; Vafeiadou et al., 2018a). Reduced body-size and biomass as well as reduced population growth have been reported for soil and freshwater bacterivorous nematode species under thermal stress as a consequence of increased metabolic rates (Majdi et al., 2019). The impacts of warming on nematode metabolic activities are also reflected in their assimilation and respiration rates, reproduction and generation times, and egg and pre-adult mortality (Moens and Vincx, 2000b). Our results are in agreement with the previous studies and validate the use of free-living aquatic nematode species for assessing effects of temperature changes.

Egg deposition of *D. delyi* was enhanced at increased temperatures (28 °C to 32 °C). This is in agreement with studies on two other *Diplolaimelloides* species (*D. meyli* and *D. oschei*), showing that egg deposition increased in treatments with constant but higher temperatures between 25 °C and 30 °C (Moens and Vincx, 2000a) and in daily fluctuating temperature regimes of 20 to 32 °C (Vafeiadou et al., 2018a). However, the lack of juvenile, and subsequently adult development at 35 °C is an indication that 35 °C exceeds the thermal tolerance threshold of *D. delyi* when exposed to this temperature continuously. This is implied by the fact that this nematode species originated from a tropical location where such extreme temperature maxima are often reached but are not persistent. Air temperature in tropical areas may exceed 40 °C, although wet sediments reach lower temperatures due to the high specific heat capacity of the pore water, allowing interstitial species to survive such extreme conditions. Similar results have been reported for other *Diplolaimelloides* species from a temperate location, with up to 100% mortality of juveniles at the 30 °C (Moens and Vincx, 2000a).

The decrease in juvenile abundances with rising temperatures despite the large numbers of eggs, means that even though egg deposition was not disrupted, the temperature increase prevented eggs from developing and hatching. This result contradicts with the observed trend of increasing juvenile and adult abundances following increasing number of deposited eggs with increasing temperatures (25–30 °C) as seen for *D. meyli* (Moens and Vincx, 2000a). However, egg mortality or disrupted egg development under thermal stress conditions has also been reported for another monhysterid species (*Halomonhystera disjuncta*), suggesting that increased temperatures may result in fast egg deposition of less-developed (and thus less-resistant) eggs (Vafeiadou and Moens, 2021, this issue). Moreover, the maximum temperature used in our experiment was relatively high compared to the temperatures used by Moens and Vincx (2000a,b), suggesting that species of the genus *Diplolaimelloides* are stenothermal, i.e. have the ability of living or surviving within a narrow temperature range. This ecological adaptation to temperature ranges seems independent of the nematodes' origin (i.e. tropical or temperate), and species may adapt to different temperature regimes. Tropical stenothermic marine nematode species normally have a higher tolerance to increased temperatures but tend to tolerate a smaller range of temperatures, whereas temperate species have a relatively higher tolerance to a larger temperature range and temperature fluctuations (less stenothermic restriction), but cannot cope with high (extreme) temperatures (Vafeiadou et al., 2018b). The congeneric *Diplolaimelloides* species, therefore, seem to tolerate a temperature range that does not exceed 32 °C or 35 °C, for the temperate and tropical species of this genus, respectively. Congeneric species from different regions also show different responses related to their origins, their habitat adaptation and evolutionary history. Nematode population growth is directly linked to fitness and their ability to cope with certain ecological conditions, such as food availability, species competition, predatory pressure and different temperature regimes (dos Santos et al., 2009; dos Santos and Moens, 2011; Vafeiadou et al., 2018a), making this stenothermic species suitable for temperature stress assays.

The non-significant variation in development times between 25 °C and 30 °C reinforces the idea that temperature variations within this range do not impact development of *Diplolaimelloides* populations (Moens and Vincx, 2000a). However, the optimal temperatures for each population development are likely related to their natural habitat (Sudhaus, 1980). This may be particularly the case for species with a narrow range of thermal tolerance, and may explain the significant differences seen at 32 °C and higher in the present study.

Both daily and total reproductive output increased at temperatures of 28 °C, 30 °C and 32 °C, in accordance with other studies that reported an increase in reproductive success of marine nematodes with increasing temperatures, but within their optimal temperature range (Heip et al., 1985; Moens and Vincx, 2000a,b; Vafeiadou et al., 2018a; Vranken et al., 1988). This can be explained as an adaptive strategy in response to warming, whereby energy load is used for reproduction to maintain population success, as seen in copepods (Stringer et al., 2012). The non-significant difference in sex ratio between different temperatures reported here has also been observed in two other *Diplolaimelloides* species (Vafeiadou et al., 2018a) and suggests that temperature increase (within the range applied) does not have an effect on sex ratio within this genus. In accordance with previous studies reporting the effects of elevated temperature on several aquatic free-living nematodes under constant and fluctuating temperature regimes (De Meester et al., 2015; Majdi et al., 2019; Vafeiadou et al., 2018a), we emphasize the importance of and need for further experimental studies using a broader variety of nematode taxa and a wider temperature range. The responses of different species to temperature variations and warming, and assessment of their thermal tolerance limits, are imperative to understand the future of meiofaunal species in a warming world.

4.3. Exposure of Diplolaimelloides delyi to different phenanthrene concentrations at increased temperature

The decrease in population growth (adults, juveniles and eggs), or even mortality, of *D. delyi* populations exposed to phenanthrene at increased temperature (30 °C) compared to those exposed to phenanthrene at control temperature (25 °C), demonstrate that non-significant concentrations at habitual temperatures can become significant and deleterious with certain increase in temperature. The synergistic effects of warming and exposure to phenanthrene on *D. delyi* were more severe than the effects of each tested stressor individually. This may be related to the modification of enzymatic activity, molecules or cellular abortion channels, among other possible causes, at increased temperatures, which makes the contaminant even more harmful to the exposed populations (Laetz et al., 2014; Schiedek et al., 2007; Zebral et al., 2019).

The addition of a non-contaminant environmental stressor such as

temperature may exacerbate the effects of low-concentration toxicants, and is considered a threat for aquatic biodiversity (Liess et al., 2016). There is evidence for commonly abundant meiofauna species being less tolerant to the combined effects of temperature and other non-contaminant environmental stressors, such as pH or salinity changes. For instance, reduced abundances of nematodes, copepods and ostracods have been observed as a combined effect of warming and ocean acidification (Ingels et al., 2018). However, nematode community structure seemed more sensitive to increased acidification and temperature than abundance *per se* (Lee et al., 2017). Copepods' sensitivity to biocides, on the other hand, is highly temperature-dependent (Bao et al., 2008). Thermal-induced toxicity has also been reported for non-meiofaunal crustaceans, the amphipod *Gammarus pulex* (Russo et al., 2018).

The significant differences in total development time of populations exposed to low phenanthrene concentrations of 1 μg ml^{-1} and 2.5 μg ml^{-1} at 30 °C, alongside the reduction as well as disappearance of all larval stages at a concentration of 5 μg ml^{-1} at 30 °C, reinforce the idea that temperature increase intensifies the effect of the contaminant on our test species. Daily and total reproductive output was significantly reduced in all populations exposed to phenanthrene regardless of the concentration, when compared to controls (no contaminant) at 30 °C. Warming in synergy with contaminant exposure negatively affects the oviposition of the species, whereas reproductive success increased with temperature increase in the absence of contaminants, even at 30 °C. The interactive effect of phenanthrene and warming is also implied by the significantly reduced reproductive output even at the lowest phenanthrene concentrations at 30 °C, whereas phenanthrene exposure of 0.1 μg ml^{-1} and 1 μg ml^{-1} at control temperature (25 °C) resulted to an oviposition equivalent to that in the controls (C and CS, no contaminant).

Most aquatic nematodes have a metabolic tipping point between 25 °C and 35 °C (Moens and Vincx, 1998; Vafeiadou et al., 2018a), with very few (soil) species tolerating higher temperatures (up to 40 °C) (Majdi et al., 2019). Our results demonstrate that temperature increase above 35 °C is unbearable for the population growth of *D. delyi*, as well as for many other nematode species (Anderson and Coleman, 1982; Venette and Ferris, 1997; Moens and Vincx, 2000a,b; Majdi et al., 2019), while the addition of phenanthrene can reduce the metabolic tipping point for *D. delyi* in synergy with thermal stress.

5. Conclusions

We highlighted the versatility of the genus *Diplolaimelloides,* more specifically the species *D. delyi,* as model organisms for rapid assessments of ecotoxicological effects. We demonstrated that labour-intensive analyses of a range of life-history traits and population parameters may be successfully replaced by a combination of reproductive output (as the most sensitive life-history trait), and juvenile and adult abundances, which integrate information of reproductive output, development time and survival.

Phenanthrene contamination had negative but non-lethal impacts on *D. delyi* populations in our single-stressor assays. Exposure to moderate-high phenanthrene concentrations negatively impacted important parameters of the species' life-history, such as population growth, development time and reproductive rates. Such effects intensified with a concurrent increase in temperature, which proved lethal for our test populations at the highest temperature (35 °C), and at high phenanthrene concentrations. Little is known, however, about the responses of other marine nematode species to these two stressors in interaction. Further studies are essential to assess the possible impacts of the synergistic effect of contaminants and temperature rise, especially in the times of an ongoing climate change and the environmental variability that comes along with it.

This study suggests that the sensitivity of marine nematodes to toxic compounds increases with warming, while the toxicity of the contaminants may also be temperature-induced. This implies that the accumulation of hazardous organic compounds in the marine environment will cause more severe effects with temperature rise. In a global change era where ocean warming is inevitable, bioaccumulation of such contaminants in higher trophic levels may also disrupt marine food webs with further implications for ecosystem functions. Further insights of the possible additive effects of pollution and temperature on a larger number of marine species and communities are crucial for policymakers. Knowledge on how currently low-impact or stable contamination levels can affect species fitness and ecosystem fuctions under warming conditions may aid in marine management strategies and preemptive action to mitigate severe impacts in the future.

CRediT authorship contribution statement

Leticia Pereira Pontes: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Anna-Maria Vafeiadou:** Writing - review & editing, Visualization. **Flavia Juliana Lobato de França:** Investigation. **Raianne Amorim Cavalcante:** Investigation. **Débora Alissandra de Araújo França:** Investigation. **Clara Moura Brito:** Writing - original draft. **Romulo Nepomuceno Alves:** Conceptualization. **Paulo Sérgio Martins de Carvalho:** Conceptualization, Visualization, Resources, Writing - original draft. **Giovanni Amadeu Paiva dos Santos:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision.

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.

Acknowledgments

LP Pontes is funded by Coordination for the Improvement of Higher Education Personnel, Brazil (CAPES). AM Vafeiadou benefitted from a PhD grant from Ghent University, Belgium (BOF) and is currently supported by the Flemish Science Fund FWO through project GOC3218N. FJL de França and DAA França were funded by the National Council for Scientific and Technological Development, Brazil (CNPq). RN Alves was funded by FACEPE Pernambuco Science and Technology Support Foundation - Brazil. Authors would like to thank the Marine Biology Lab at Ghent University, Belgium, for keeping available and reproducing the nematode culture used in this study as part of their culture collection that is maintained as part of infrastructure of EMBRC Belgium, funded by FWO project GOH3817N. The authors would also like to thank the editors of this special issue "*Meiofauna in a changing world*", the two anonymous reviewers for their constructive comments that helped improve this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106868.

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