Ecotoxicology and Environmental Safety 141 (2017) 298–305

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Toxic effects of polyethylene terephthalate microparticles and Di(2-ethylhexyl)phthalate on the calanoid copepod, *Parvocalanus crassirostris*

Franz M. Heindler[a,*,1], Fahad Alajmi[a,b], Roger Huerlimann[a], Chaoshu Zeng[a,c], Stephen J. Newman[d], George Vamvounis[e], Lynne van Herwerden[a,c,f]

[a] College of Marine & Environmental Sciences, James Cook University, 1 James Cook Drive, Townsville, Australia
[b] Environment Public Authority Kuwait, P. O. Box 24395, Safat 13104, Kuwait
[c] Centre of Sustainable Tropical Fisheries and Aquaculture, James Cook University, 1 James Cook Drive, Townsville, Australia
[d] Western Australian Fisheries and Marine Research Laboratories, Department of Fisheries, Government of Western Australia, P.O. Box 20, North Beach, Western Australia 6920, Australia
[e] College of Science, Technology and Engineering, James Cook University, 1 James Cook Drive, Townsville, Australia
[f] Comparative Genomics Centre, James Cook University, 1 James Cook Drive, Townsville, Australia

ARTICLE INFO

Keywords:
Zooplankton
Microplastic
PET
qPCR
DEHP
Pollution

ABSTRACT

Large amounts of plastic end up in the oceans every year where they fragment into microplastics over time. During this process, microplastics and their associated plasticizers become available for ingestion by different organisms. This study assessed the effects of microplastics (Polyethylene terephthalate; PET) and one plasticizer (Di(2-ethylhexyl)phthalate; DEHP) on mortality, productivity, population sizes and gene expression of the calanoid copepod *Parvocalanus crassirostris*. Copepods were exposed to DEHP for 48 h to assess toxicity. Adults were very healthy following chemical exposure (up to 5120 µg L^{-1}), whereas nauplii were severely affected at very low concentrations (48 h LC_{50}value of 1.04 ng L^{-1}). Adults exposed to sub-lethal concentrations of DEHP (0.1–0.3 µg L^{-1}) or microplastics (10,000–80,000 particles mL^{-1}) exhibited substantial reductions in egg production. Populations were exposed to either microplastics or DEHP for 6 days with 18 days of recovery or for 24 days. Populations exposed to microplastics for 24 days significantly depleted in population size (60 ± 4.1%, $p < 0.001$) relative to controls, whilst populations exposed for only 6 days (with 18 days of recovery) experienced less severe depletions (75 ± 6.0% of control, $p < 0.05$). Populations exposed to DEHP, however, exhibited no recovery and both treatments (6 and 24 days) yielded the same average population size at the termination of the experiment (59 ± 4.9% and 59 ± 3.4% compared to control; $p < 0.001$). These results suggest that DEHP may induce reproductive disorders that can be inherited by subsequent generations. Histone 3 (*H3*) was significantly ($p < 0.05$) upregulated in both plastic and DEHP treatments after 6 days of exposure, but not after 18 days of recovery. *Hsp70-like* expression showed to be unresponsive to either DEHP or microplastic exposure. Clearly, microplastics and plasticizers pose a serious threat to zooplankton and potentially to higher trophic levels.

1. Introduction

The world plastic production increased drastically in the last two decades (Galgani et al., 2015). Large amounts of these plastics end up in the ocean. Microplastics in the ocean can be classified into two major groups: Primary microplastics, that were deliberately produced as small particles for usage (e.g. microbeads in facewash) that reach oceans through wastewater and secondary microplastics that are created through break down from larger plastic particles into micro particles through hydrodynamic processes (Chown et al., 2012; Cole et al., 2011; Wright et al., 2013). Once in the ocean, both, primary and secondary microplastics become potentially available for zooplankton to ingest (Cole et al., 2013; Desforges et al., 2015). During the fragmentation process of plastics, different plasticizers might leach out into the water column where they can have endocrine disrupting effects on organisms (Manikkam et al., 2013; Park and Gan, 2014; Rochman et al., 2014).

While microplastic ingestion by zooplankton has been confirmed in various studies, few have quantified their biological effects on organisms (Cole et al., 2015, 2013; Kaposi et al., 2014; Lee et al., 2013). In particular, potential long-term effects of these microplastics and

* Corresponding author.
E-mail address: franzmaximilian.heindler@kuleuven.be (F.M. Heindler).
[1] Present Address: Laboratory of Biodiversity and Evolutionary Genomics, KU Leuven, P.O. Box 2439, 3000 Leuven, Belgium.

http://dx.doi.org/10.1016/j.ecoenv.2017.03.029
Received 25 July 2016; Received in revised form 21 February 2017; Accepted 21 March 2017
0147-6513/ © 2017 Elsevier Inc. All rights reserved.

associated plasticizers on the reproductive output of zooplankton have not been addressed. Many plasticizers are believed to have transgenerational epigenetic effects on organisms, that is, an altered phenotype is expressed in the absence of mutational change (Gray et al., 2000; Manikkam et al., 2013; Youngson and Whitelaw, 2008). Epigenetic mechanisms allow offspring of organisms to cope and respond to environmental stress by altering the expression of relevant gene(s) in anticipation of a stressful environment to be encountered, following exposure of the parental (F_0) generation to particular stress. This is in contrast to adaption by natural selection on mutated genotypes, which was formerly believed to be the only way for species to adapt to changing environments (Jaenisch and Bird, 2003). While transgenerational mechanisms have many positive attributes, for example rapid acclimation to warming oceans by a reef fish (Veilleux et al., 2015), they could also have disadvantageous effects. Particularly, many endocrine disruptors cause the expression of disease phenotypes (Anway and Skinner, 2006; Singh and Li, 2012). Under certain circumstances such disease phenotypes can be passed on to subsequent generations transgenerationally (Skinner et al., 2011). Consequently, offspring that were not exposed to the environmental toxin themselves, can express the same disease phenotype as the parental generation. Many plasticizers have been reported to have such transgenerational effects on reproductive capacity, which may have deleterious effects on populations (Anway et al., 2005; Anway and Skinner, 2006; Zama and Uzumcu, 2010). Such transgenerational effects can be detected by assessing the expression of stress response genes. In this study, the expression of Heat Shock Protein 70 - like (*Hsp70-like*) and Histone 3 (*H3*) sequences were assessed. Both genes have shown to be linked to stress (chemical) and have shown to be relevant in other comparable studies (Rhee et al., 2009; Singh and Li, 2012). Heat Shock Proteins, a group of proteins involved cell repair through external stressors and Histones, a group of proteins that are involved in packaging and ordering DNA as well as regulating gene expression, have been previously used to show a proof-of-concept in epigenetic studies (Agnew, 2000; Singh and Li, 2012).

This study focused on Polyethylene terephthalate (PET) microplastics and the plasticizer Di(2-ethyhexyl)phthalate (DEHP), which is one of the most common plasticizers used in the plastic industry (Latini et al., 2010). PET is generally regarded as food safe and found in preliminary analysis to have negligible levels of plasticizers present out of ten plastics tested (Supporting information 1). DEHP, like many other plasticizers, generally shows low acute toxicity. However, it acts as an endocrine disruptor and has been reported to have transgenerational effects on rats, most commonly exerting negative effects on the development of sexual organs and reproductive behavior (Manikkam et al., 2013; Singh and Li, 2012; Wu et al., 2010). With high abundance of DEHP in marine environments (Table 1), especially in river estuaries and coastal areas, it is important to identify what effects it may have on the zooplankton community - the crucial link between primary producers and higher trophic levels of the marine foodweb. *Parvocalanus crassirostris* was used as a study species due to its abundance in tropical waters and its ecological role as a prey item for larval fish (McKinnon and Ayukai, 1996; McKinnon and Duggan, 2014; McKinnon et al., 2005). In this study the effect of Di(2-ethylhexyl)phthalate and PET microparticles on the survival, fertility (one generation), relative population size (prolonged exposure) and gene expression (*H3*, *Hsp70-like*) in *P. crassirostris* were assessed.

2. Material and methods

2.1. General procedures

Parvocalanus crassirostris were maintained at James Cook University (since 2012), Australia and were fed with *Isochrysis* sp. Maintenance of both cultures and experimental conditions followed the procedures described in detail in Alajmi and Zeng (2013). In short, cultures were kept in 20 L polycarbonate carboys at 26 ± 1 °C, filled with 1 µm filtered seawater and a photoperiod of 16:8 h (light:dark) at a light intensity of1400 lx during the light phase. Carboys were cleaned and partial water changes were performed on a weekly basis. Reported development time from hatching to adulthood was 6.2 days (± 0.1 days) at a temperature of 26 °C (± 1 °C) when fed with the phytoplankton, *Isochrysis* sp. (T-iso). Development time is highly variable depending on food availability and quality as well as temperature. Accordingly, standardization of methods is crucial in order to normalize development time. Population densities are stable at up to 5 adult individuals m L^{-1} (Alajmi and Zeng, 2013). Bis(2-ethylhexyl)phthalate (DEHP) was obtained from Sigma-Aldrich (Pestanal®, analytical standard) and dissolved in filtered (1 µm) seawater (36‰). Concentrations of *Isochrysis* sp. were determined using a haemocytometer and added to all treatments and controls at a concentration of 20,000 cells m L^{-1}. Microplastic particles were derived from conventional Polyethylene terephthalate (PET) water bottles (Woolworths Select Mountain Still Spring Water; Woolworths Pty Ltd, Australia). PET was ground up using sandpapers. Distilled water was added during the process to prevent PET particles from becoming airborne. The density difference between sand and PET was utilized to separate waterborne plastic particles from potentially detached settled sand grains, generated by using sandpaper to shred PET bottles. Particles were filtered through three sieves (100, 25 and 11 µm) to isolate particles in the size range of *Isochrysis* sp. (5–10 µm). The shape of microplastics particles produced were edged as described previously of particles found in the environment (Supporting information 2)(Wagner et al., 2014). PET particles were then mixed with seawater (36‰) to obtain the desired concentrations (Table 2b) based on a microplastic to food ratio of 1:1, 2:1, 4:1 and 8:1.

Table 1
Observed and predicted (*) environmental concentrations (in µg L^{-1} and ng L^{-1}) of Bis(2-ethylhexyl)phthalate in aquatic systems. Where multiple locations were assessed, the average (ø) of all measurements from the study was used.

Concentration k		Location	Citation
µg/L	ng/L		
ø 0.5	ø 500	Annual Average of 26 locations of fresh and marine waters, Netherlands	Peijnenburg and Struijs (2006)
1.3*	1300*	European norm for surface water (2007)	Dargnat et al. (2009)
2.2*	2200*	Predicted Environmental Concentration	Fromme et al. (2002)
ø 2.27	ø 2270	Marine surface waters, Germany	Fromme et al. (2002)
4.6	4600	Tap water, Montreal, Canada	Horn et al. (2004)
ø 8.8	ø 8800	Sewage effluents (Germany)	Fromme et al. (2002)
9–44	9000–44.000	Wastewater, Marne Aval, France	Dargnat et al. (2009)
47	47,000	Creek water, Montreal, Canada	Horn et al. (2004)
58	58,000	Freshwater, Japan	Naito et al. (2006)
62	62,000	Landfill leachate, Montreal Canada	Horn et al. (2004)
180	180,000	River water, Montreal, Canada	Horn et al. (2004)

2.2. Toxicity test

Preliminary tests for adults and nauplii have been conducted to determine the scope of concentrations reaching from no effect to 100% mortality (Supporting information 3).

2.2.1. Adults

Standard static renewal tests were adopted for the acute toxicity test of adults (Weber, 1991). As the sex ratio of *P. crassirostris* is highly skewed towards females (> 85%) and males do not feed after maturation, only females were used for the experiment (Alajmi and Zeng,

Table 2a
Concentration of Di(2-ethylhexyl)phthalate (ng mL^{-1} or ng L^{-1}) inoculated dietary supplements to feed the copepod *Parvocalanus crassirostris* for Sections 2.3 and 2.4. All treatments contain *Isochrysis* sp. at 20 000 cells mL^{-1}.

Experiment (Section)	Treatment	Dietary Supplement	Concentration $ngmL^{-1}$ or ng L^{-1}	DEHP to algal cell ratio
2.3 + 2.4	Control	–	–	–
2.3	D1	DEHP	0.33 ng mL^{-1}	–
2.3	D2	DEHP	1.00 ng mL^{-1}	–
2.3	D3	DEHP	3.00 ng mL^{-1}	–
2.4	D4	DEHP	0.110 ng L^{-1}	–

Table 2b
Concentration of microplastic particles (particles ml^{-1}) as dietary supplements to feed the copepod *Parvocalanus crassirostris*. All treatments contain *Isochrysis* sp. at 20,000 cells $\mathbf{mL^{-1}}$. The ratios of microplastic particles to algal cells were calculated based on the standard 20,000 cells $\mathbf{mL^{-1}}$ of *Isochrysis* sp. provided to feed *P. crassirostris*.

Experiment (Section)	Treatment	Dietary Supplement	Concentration (particles mL^{-1})	Microplastic to algal cell ratio
2.3 + 2.4	Control	–	–	–
2.3	P1	Microplastic	10,000	0.5:1
2.3	P2	Microplastic	20,000	1.0:1
2.3	P3	Microplastic	40,000	2.0:1
2.3	P4	Microplastic	80,000	4.0:1
2.4	P5	Microplastic	20,000	1.0:1

2013). To ensure uniform age of experimental animals, the final copepodite stage (C5, length 389.9 ± 1.1 μm) (Alajmi et al., 2015) of *P. crassirostris* were identified and collected from the stock cultures 24 h prior to the experiment using a developmental key (Lawson and Grice, 1973). 250 individuals were transferred to an aerated 500 ml glass jar and left for 24 h without food to let them reach sexual maturity and acclimate to experimental conditions. To start the experiment, 10 females were randomly selected and their sexual maturation confirmed. They were then placed in 25 ml glass jars with 20 ml of one of six DEHP experimental solutions at 160 μg L^{-1}, 320 μg L^{-1}, 640 μg L^{-1}, 1280 μg L^{-1}, 2560 μg L^{-1} and 5120 μg L^{-1}. For high concentrations (> 1280 μg L^{-1}) ethanol was added to the seawater (4%) to increase solubility. A control group with ethanol was implemented. For each treatment (6 concentrations) and control (2 controls: with and without 4% ethanol), 5 replicates were maintained. All replicates were placed on a plankton wheel with a rotation rate of 0.5 rotations per minute to keep algal cells in constant suspension in order to simulate natural conditions. After 24 h, all samples were poured into petri dishes in order to assess copepod survival in each replicate using a dissecting microscope. If no movement was observed after 1 min, individuals were recorded as dead. Surviving individuals were returned to their respective jars for another 24 h with fresh test solution added as before, including fresh *Isochrysis* sp. The experiment was terminated after 48 h when the survival for each replicate was re-assessed.

2.2.2. Nauplii

The experimental procedures were similar to those described for adult copepods (2.2.1). For each treatment 180 nauplii from the first 3 nauplii stages (N1-3, length 80 ± 0.6 – 112.1 ± 0.4 μm) (Alajmi et al., 2015) were obtained from the stock culture and placed in 25 ml glass jars for 48 h with lowered DEHP concentrations due to increased mortality of nauplii compared to adults (0.06 ng L^{-1}, 0.48 ng L^{-1}, 3.81 ng L^{-1}, 20.52 ng L^{-1}, 244.14 ng L^{-1} and 1953.13 ng L^{-1}). For each treatment, 3 replicates were conducted. Nauplii were obtained by gently filtering copepod stock cultures through a 74 μm mesh, which retained adults and copepodites, allowing nauplii to be collected subsequently on a 25 μm mesh. To maintain uniformity in sampling design, *Isochrysis* sp. was added to a concentration of 20,000 cells L^{-1}, despite nauplii being non-feeding (Mauchline, 1998).

2.3. Reproductive output of *P. crassirostris* in one generation

For the assessment of the effects of dietary microplastic and DEHP supplements on egg production, *P. crassirostris* individuals were exposed to one of seven treatments of either no exposure (control), plastic exposure or DEHP exposure, as described in Table 2a (P1, P2, P3) or Table 2b (P4, D1, D2, D3), respectively. For each treatment, 10 replicates of 20 female and 4 male *P. crassirostris* were prepared, which is the natural ratio (Alajmi and Zeng, 2013). Premature stage copepodites (C5) were collected from *P. crassirostris* stock cultures and males and females were initially separated 24 h prior to commencing the experiment and placed into an aerated 5 L glass container supplied with fresh seawater without food. Stage 5 copepodites molt and become adults within 24 h (Alajmi and Zeng, 2013), the newly matured adult copepods were then transferred into 20 ml glass jars containing the relevant test solution and placed on a plankton wheel rotating at 0.5 rotations per min to ensure micro PET particles and microalgae remained suspended. Adult *P. crassirostris* were exposed to different treatments for 5 days. Complete (100%) water changes were conducted on day 3 to restore initial conditions. After 5 days, the total content of each jar was filtered sequentially through a 150 μm mesh to retrieve adults (~300 μm), whilst nauplii (40–100 μm) and eggs (~60 μm) were subsequently recovered by filtration of the eluate through a 25 μm mesh. Because none of the offspring would have reached adult stage during the 5 day experimental period, the 150 μm mesh collected only the initially stocked large adults (i.e. 20 females and 4 males). All stages of *P. crassirostris* collected were then assessed under a light microscope and the number of offspring recorded. Naupliar and copepodite stages were counted as eggs produced by females, in addition to any eggs recovered. Furthermore, the number of females was assessed and mortality was taken into account when calculating productivity.

2.4. Relative population size of *P. crassirostris* over prolonged exposure

All treatments contained 4 replicates, each of which was initially stocked with 36 individuals between copepodite (C1) and adult stages, as well as 68 nauplii, as this represents a natural ratio observed in healthy stock cultures. *Parvocalanus crassirostris* used for each replicate were randomly harvested from the stock cultures and stocked in 270 ml plastic jars filled with 210 ml of test solution (natural seawater only for the control). Test solutions were prepared as either microplastics at concentrations of 20,000 particles mL^{-1}(Table 2a; P5; a 1:1 ratio of microplastic particles:microalgal cells) or DEHP at 0.11 ng L^{-1}(Table 2b; D4), depending on the treatment. DEHP concentrations of 0.11 ng L^{-1}, which is approximately 10% of the 48h – LC_{50} concentration (Section 3.1) for *P. crassirostris* nauplii (1.04 ng L^{-1}), were adopted to ensure sufficient numbers of surviving offspring for the prolonged exposure experiments.

Average development time from hatching to adulthood is 6.2 ± 0.1 days and average adult lifespan is 8.8 ± 0.1 days under given culture conditions (Alajmi and Zeng, 2013; Alajmi et al., 2015). Individuals were not removed during the experiment. At the termination of the experiment after 24 days there will be a mix of the first and second offspring generation and all of the initially stocked individuals will have died. As such, populations of *P. crassirostris* were subjected to each of the following five treatments for 24 days, in order to assess potential transgenerational effects of microplastics and DEHP on reproductive capacity. Treatments were: 1) Neither microplastics nor DEHP was added (control treatment); 2) microplastics were added at 20 000 particles ml^{-1} for 6 days, followed by a recovery period without added microplastics (complete water change) for the remaining 18 days; 3) microplastics were added at 20 000 particles ml^{-1} for the 24 day experimental duration; 4) DEHP was added at 0.11 ng $L^{-1}$for 6 days

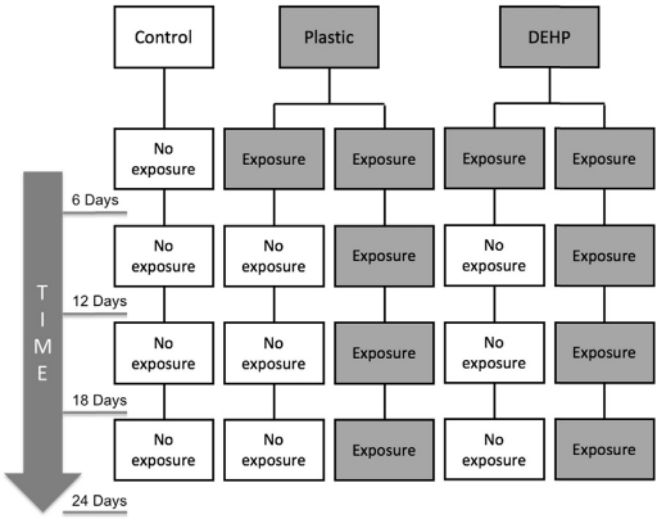

Fig. 1. Schematic of experimental design showing treatments in which *P. crassirostris* were exposed to microplastics or DEHP. Population size was assessed at six day intervals. For each treatment four replicates were conducted. White and gray coloring indicates no exposure or exposure to the specified plastic or DEHP treatment, respectively.

followed by a recovery period without any additives (complete water change) for 18 days and 5) DEHP was added at 0.11 ng $L^{-1}$for the 24 day experimental duration (Fig. 1).

All replicate jars for all treatments were sealed and placed on a plankton wheel rotating at 0.5 rotations min^{-1}. After 3 days microalgae was added at a concentration of 20,000 cells $mL^{-1}$to prevent starvation. After 6 days, the content of each replicate jar was sieved through a 25 µm mesh to retain all life stages, including eggs, but not plastic particles or DEHP, and then carefully rinsed into a Bogorov tray. The number of adults/copepodites as well as nauplii was assessed separately. Jars were rinsed, refilled with fresh test solution and *Isochrysis* sp. feed at 20,000 cells mL^{-1}, whereafter all stages of *P. crassirostris* were returned from the Bogorov tray by careful rinsing. This procedure was repeated 4 times (every 6 days), until day 24 when the experiment terminated. At every stage the number of individuals was determined. After 24 days, based on development and survival time, we would have a mixture of the first and second offspring generation of the initially stocked individuals.

2.5. Gene expression

Approximately 500 individuals were placed in 15 L plastic carboys with fresh seawater (control) or fresh seawater containing either PET particles or DEHP (treatments) at a concentration of 20,000 particles L^{-1} and 0.1 ng L^{-1}, respectively (as per culture procedures described in 2.4). For each treatment and control 3 replicates were conducted. *Isochrysis* sp. was added daily at concentrations of 20,000 cells ml^{-1} during the 24 days of the treatment to ensure that food was not limiting. In order to assess the transgenerational effects of PET particles or DEHP exposure, populations were exposed to either treatment for 6 days, followed by a recovery period of 18 days. On day 6 of the experiment individuals from each replicate, inducing controls, were siphoned out and collected on a 25 µm mesh. Individuals were then gently placed in a 30 ml seawater solution. Carboys were cleaned and filled with fresh seawater and half of the individuals (15 ml) were gently placed back into their original carboys. The remaining half of the individuals were collected on a 25 µm mesh and rinsed with RNAlater for later evaluation of gene expression. These copepods were flushed with RNAlater into 2 ml tubes and stored at 4 °C for 24 h after which they were processed. After another 18 days the experiment was terminated and all individuals were siphoned out, collected on a 25 µm mesh, rinsed with RNAlater, stored in 2 ml of RNAlater at 4 °C for 24 h and then processed.

Extraction of RNA from pooled samples of each replicate of day 6 and day 24 was performed using TRIzol® (Thermo Fisher, Catalogue # 15596-026) and followed a modified version of the manufacturer's protocol based on Chomczynski and Sacchi (1987). Extracted RNA was treated with the TURBO DNA-free™ Kit (Thermo Fisher; Cat. #AM1907) to remove potential DNA and proteins. Synthesis of cDNA was performed by using the commercial iScript™ cDNA synthesis kit (Bio-rad, Catalogue #1708890) with a total amount of 500 ng RNA. The genes *18S* (NCBI Accession Nr: KU861810) and *COI* (NCBI Accession Nr: KU933944) were used as housekeeping genes, as they have previously exhibited relatively stable expression in humans, mice and shrimps for *18S* (Feng et al., 2014; Vartiainen et al., 2011) and fish for *COI* (Laver and Taylor, 2011). We expected variance in expression of *H3* NCBI (Accession Nr: KU933943) and *Hsp70-like* NCBI (Accession Nr: KU933945) based on previous studies (Rhee et al., 2009). Real time quantitative polymerase chain reaction (RT-qPCR) primers were designed using Geneious® 8.1.5 (Kearse et al., 2012) (Supplementary information) and ordered from GeneWorks, Adelaide. Gene expression was determined using SsoFast™ EvaGreen® Supermix Kit (Bio-rad, Catalogue #1725201), following manufacturer protocols in a Thermocyler (Rotor-Gene Q, Qiagen). The amplification program consisted of heating the samples to 95 °C, followed by 40 cycles of 95 °C for 5 s, 59 °C for 20 s. The melting curves were obtained by heating the samples from 59 °C to 95 °C in 0.5 °C/s increments. Each sample was run in triplicate for quality control purposes of quantitative PCR.

2.6. Statistical analysis

Data on survival, reproductive output and relative population size were presented as mean ± SE. R Studio with R v3.1.2 (R Studio®, Package: Agricolae, R Core Team, 2015) was utilized for statistical analysis. Survival, reproductive output and population size of copepods in each treatment were adjusted to the relevant control in order to account for natural mortality, productivity and population size throughout the experiments. For relative population size the average of all counts post exposure (day 6, 12, 18 and 24) were taken to reduce noise. After testing for normality and homogeneity of variance, a one-way ANOVA and Tukey's post hoc analysis was employed to test for and identify significant differences among treatments.

Quantitative RT-qPCR analysis was performed employing a model that assumed fixed (treatment and generation) and random (individual) effects and compared the expression of each gene in turn for both exposure treatments (Plastic or DEHP), to the expression of that gene in the control group for the assessment after 6 and 24 days (Package CMCM.qpcr). The NormFinder algorithm was used to determine the best combination of housekeeping genes (Andersen et al., 2004). Variance of cDNA quantities was accounted for by allowing for Bayesian variation within replicate treatments. Significance levels were obtained by performing a z-test on the Bayesian z-score in order to acquire a two-tailed p-value and was corrected for multiple testing (Benjamini and Hochberg, 1995; Matz et al., 2013).

3. Results

3.1. Toxicity test

For adults, there were no significant differences between treatments and controls. There were also no significant differences between controls (with and without ethanol). An LC_{50} could not be established since even at the highest DEHP concentration (5120 µg L^{-1}), the mortality rate did not exceed 8.7 (SE ± 7.1)%. The highest mortality of 18.3 (± 9.2)% was observed at a concentration of 2560 µg $µL^{-1}$. Mortalities of different treatments fluctuated with no significant correlation to DEHP concentration ($p > 0.05$).

Survival of *P. crassirostris* nauplii proved to be highly sensitive to Di (2-ethyhexyl)phthalate, despite being exposed to more than 1000-fold

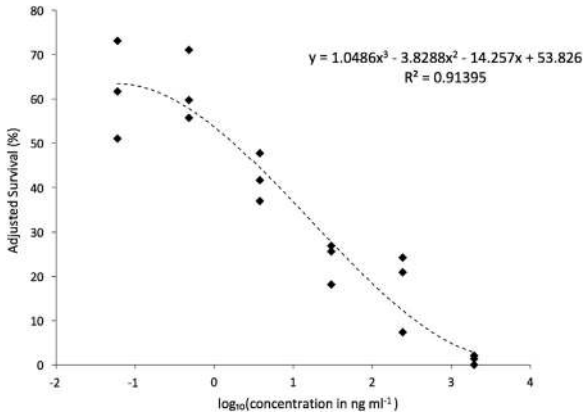

Fig. 2. Adjusted survival of *P. crassirostris* nauplii after 48 h of exposure to Di(2-ethylhexyl)phthalate. DEHP concentrations were $\log_{10}$ transformed. Concentrations prior to the transformation (from left to right) were 0.06, 0.48, 3.81, 30.52, 244 and 1953 ng L^{-1}. Diamonds represent replicates.

lower concentrations than adults. All treatments had significantly higher mortalities than the control ($p < 0.05$, Fig. 2). Average nauplii survival for the control was 82.6 (SE ± 5.1)%. At the highest DEHP concentration of 1953 ng L^{-1}, there were few surviving nauplii after 48 h exposure (1.12%; SE ± 0.4). The 48 h LC_{50} was determined to be 1.04 ng L^{-1}. Concentration proved to be a highly significant predictor of mortality ($p < 0.05$). The sigmoidal model used to determine the naupliar LC_{50} value accounted for 91% of the variation of the data, with an R^2 value of 0.9139.

3.2. Reproductive output of *P. crassirostris* in one generation

There was a negative correlation between reproductive output of *P. crassirostris* and exposure to either microplastic particles or DEHP (Fig. 3). The average egg production per female for the control over the duration of the experiment (day 5) was 1.10 ± 0.14 eggs. This is much lower than *P. crassirostris* egg production previously reported, with a daily egg production of up to 27.4 ± 0.9 eggs per female per day (Alajmi and Zeng, 2013, 2014). Low egg production is likely due to handling stress. With increasing microplastic abundance, egg production decreased and there was a negative correlation ($R^2 = 0.56$) between egg production and concentration of microplastic exposure (Fig. 3a). Despite a clear decreasing trend, differences in egg production between treatments with different microplastic concentrations (10,000, 20,000 and 40,000 particles ml^{-1}, i.e. microplastics:phytoplankton ratios of 0.5:1, 1:1 and 2:1) were not significantly different to control (0.99 ± 0.12, 0.92 ± 0.08 and 0.56 ± 0.09 eggs $female^{-1}$, respectively, $p > 0.05$). However, when microplastic concentration was 80,000 particles ml^{-1}, i.e. a microplastics:phytoplankton ratio of 4:1, egg production dropped sharply to 0.13 ± 0.05 eggs $female^{-1}$, which was significantly lower than in all other treatments ($p < 0.001$) (Fig. 3a) and 88% lower than egg production in the control treatment.

Exposure to DEHP had significant effects on *P. crassirostris* egg production, which dropped steeply by 95% from 1.10 ± 0.14 eggs $female^{-1}$ in control to 0.05 ± 0.01 eggs $female^{-1}$ following exposure to the lowest dose of 0.3 ng mL^{-1} DEHP ($p < 0.001$, Fig. 3b). As DEHP concentration increased to 1.0 and 3.0 ng mL^{-1}, egg production remained at 0.05 (± 0.03 and ± 0.13, respectively) eggs $female^{-1}$, which was not significantly different among the three DEHP exposure treatments ($p > 0.05$).

3.3. Relative population size of *P. crassirostris* over prolonged exposure

Relative mean population size of *P. crassirostris* was substantially affected by exposure to either microplastics or DEHP. Mean relative population sizes (i.e. expressed as % of the control population size) of

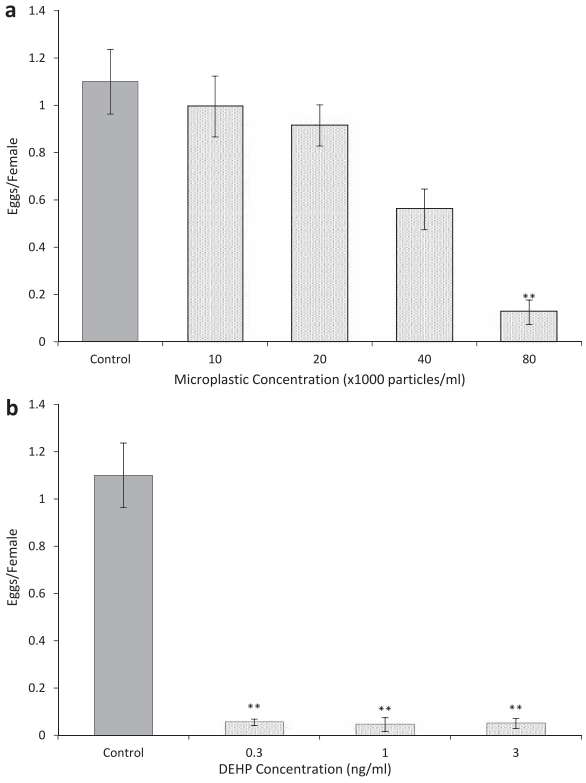

Fig. 3. Recorded egg (mean ± SE; n = 10) per female *Parvocalanus crassirostris* at the end of day five, after 5 days of exposure to different concentrations of a) microplastics and b) DEHP. Levels of significance compared to control are indicated by asterisks (*) above the bars: (*) $p < 0.05$ and (**) $p < 0.001$.

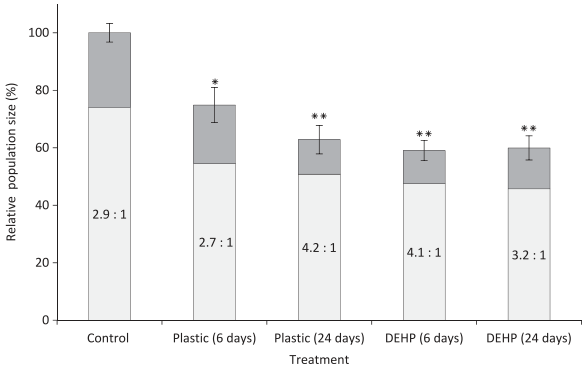

Fig. 4. Relative *Parvocalanus crassirostris* population size (mean ± SE, n = 4) over 24 days of culture under different treatment conditions in which the initial *P. crassirostris* populations were either exposed to microplastic or DEHP for 6 days only or for 24 days, respectively. Population sizes were measured every 6 days (Day 6, 12, 18 and 24) and adjusted to % of the control population size. Upper (dark gray) part of the bars indicate nauplii and lower (light gray) indicate adult and copepodite relative population size. The mean ratio of adults and copepodites to nauplii is given within the bar for each treatment. Levels of significance compared to control are indicated by asterisks above bars: (*) $p < 0.05$ and (**) $p < 0.001$.

24 days of culture under all microplastic or DEHP exposure conditions were significantly lower than in control conditions (Fig. 4). Specifically, the average final relative population size after 6 days of micro PET exposure differed from the control population size, $p < 0.05$ (75 ± 6.0% of the control population); continuous exposure to microplastics for 24 days led to highly significant population size reductions ($p < 0.001$) (60 ± 4.1% of control populations) (Fig. 4). Likewise, both DEHP exposures for 6 and for 24 days produced similarly reduced, highly significant final average population sizes of 59 ± 4.9% and 59 ± 3.4% relative to control ($p < 0.001$). Amongst the four treatments in which *P. crassirostris* were exposed to either microplastic or DEHP,

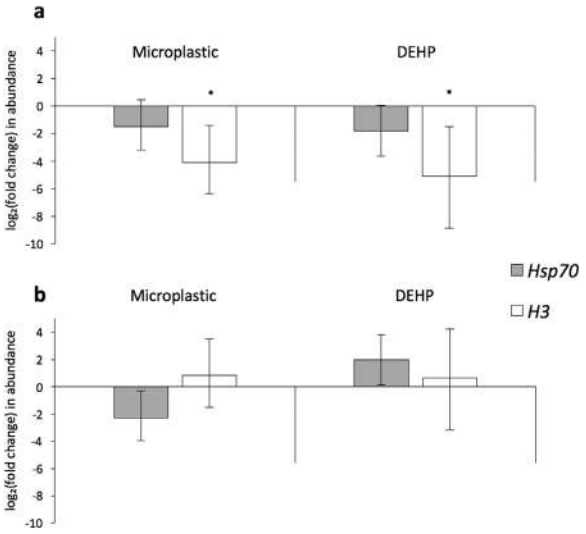

Fig. 5. Mean $\log_2$[fold change] in gene expression (± SE; n = 3) of *Hsp70-like* and *H3* in either plastic or DEHP exposed treatments (following normalization to housekeeping genes and relative to expression of these genes in the control group): **a)** expression 6 days of exposure and **b)** expression on day 24 (after 18 days of recovery), in each respective treatment. Asterisk (*) indicate significant gene expression differences compared to control at $p < 0.05$(*).

the average population size of 24 days was greatest for the 6 day microplastic exposure treatment. No significant differences were detected between the four treatments ($p > 0.05$). The ratio of adults and copepodites compared to nauplii was greater in the control (2.9:1) and plastic treatment for 6 days (2.7:1) then it was for the other treatments (plastic for 24 days: 4.2:1; DEHP for 6 days: 4.1:1 and DEHP for 24 days: 3.2:1).

3.4. Gene expression

Data are represented as $\log_2$ (fold change) in mean expression of either *Hsp70-like* or *H3* in treatments compared to the expression of the same genes in control groups (including 95% confidence intervals, Fig. 5). Expression of *Hsp70-like* and *H3* were referenced to the geometric mean of the expression of *18 S* and *COI* (Matz et al., 2013). There was no change of expression of *Hsp70-like* observable for neither treatment (plastic; DEHP) nor time assessed (6 or 24 days; Fig. 5a, b). *H3* gene expression was more responsive and was significantly down regulated after 6 days of exposure in both treatments: microplastics (−4.1, 95% CI [−6.4 to −1.4], $p < 0.05$) and DEHP (−5.1, 95% CI [−8.9 to −1.5], $p < 0.05$) (Fig. 5a). No significant *H3* expression changes were detectable on day 24 after 18 days of recovery of both plastic and DEHP exposed treatments (Fig. 5b).

4. Discussion

4.1. Microplastics

Together, results from the experiments in this study provide strong evidence for the negative effects of microplastics on the reproductive output of *P. crassirostris*, which eventually led to population declines of the experimental populations. The negative effects of microplastic exposure found in this study are in agreement with previous studies investigating microplastic ingestion by zooplankton which resulted in decreases in egg size and hatching success of the marine copepod *Calanus helgolandicus* (Cole et al., 2015). However, no significant changes in egg production were reported in the aforementioned study. The authors attributed decreased egg size and hatching success to impeded feeding by microplastic ingestion, which led to decreased energy intake. In recent studies by Cole et al. (2013) and Cole et al. (2015), round polystyrene microbeads (20 μm) were used for their experiments, which proved to be readily excreted by copepods after exposure ceased. Since microplastic particles in the environment are irregularly shaped and sometimes sharp edged (Fischer et al., 2015), the microplastic particles used in the present study were purposely produced to resemble such features (Supporting information 2). Due to their irregular surface, such microplastic particles may further complicate digestion, leading to more severe blockages in, and possible damage to digestive tracts. The prolonged exposure experiment indicated that population sizes after microplastic exposure were significantly reduced. However, mean population size at the end of a culture period spanning 24 days was greater when *P. crassirostris* were exposed for only 6 days, than when continuously exposed for 24 days. This result suggests that population recovery from microplastic exposure of 6 days would take more than 18 days to recover to pre-exposure levels, which equals to 1–2 generations after exposure. There was no evidence of microplastic exposure having transgenerational effects on reproductive systems in relation to fertility or egg production on subsequent generations.

4.2. DEHP

The $48hLC_{50}$ for adult *P. crassirostris* exposed to Di(2-ethylmehtyl) phthalate could not be established. Seo et al. (2006) reported a $48hLC_{50}$ DEHP dose of 7–10 mg $L^{-1}$for the benthic harpacticoid copepod *Tigriopus japonicus*, suggesting that lethal doses for adult copepods may be in the 10^{-3}gram (mg) rather than the 10^{-6} gram (μg) range used in this study. However, in contrast to adults, *P. crassirostris* naupliar survival was significantly affected by DEHP even though nauplii were exposed to much lower doses than adults. This suggests that DEHP toxicity is highly variable, depending on developmental stage. Similar results of developmental stage dependent toxicity were reported for the estuarine copepod *Eurytemora affinis* (Forget-Leray et al., 2005). *E. affinis* nauplii were also the more sensitive life stage compared to adults, suggesting that copepod nauplii generally exhibit significantly greater sensitivity to DEHP in the water column than adults. DEHP toxicity was also shown to be highly species-specific: Forget-Leray et al. (2005) documented a $96hLC_{50}$ value of 511 μg L^{-1} for naupliar *E. affinis*, exceeding by more than 5 orders of magnitude the observed $48hLC_{50}$value of 1.04 ng L^{-1} for *P. crassirostris* nauplii reported here. Furthermore, female *E. affinis* exposed to half the naupliar observable effect concentration (NOEC; 109 μg L^{-1}) produced offspring that upon continuous exposure (at half the NOEC) exhibited developmental inhibition and never reached the copepodite stage, ultimately dying. These results highlight the dangers of transgenerational effects of DEHP on subsequent generations of copepods, which was also noted in Manikkam et al. (2013), where inheritance of reproductive diseases of rat offspring were reported after exposure of parents to plastics in association with DEHP.

Furthermore, DEHP significantly affected both egg production after 5 days of exposure and mean population over prolonged exposure. Exposure significantly lowered egg production at all concentrations tested (0.3, 1.0 and 3.0 ng mL^{-1}), well below what affects survival of adult *P. crassirostris*. While DEHP exposure leading to decreased reproduction capacity has been well documented in terrestrial (Gray et al., 2000; Manikkam et al., 2013) and aquatic (Uren-Webster et al., 2010; Van Wezel et al., 2000; Ye et al., 2014) vertebrates, past research produced contradictory results for zooplankton. For example, Zhao et al. (2009) found no significant effect on reproductive output of the freshwater rotifer, *Brachionus calyciflorus,* when exposed to DEHP concentrations up to 5000 ng mL^{-1}. Similarly, no significant effect on fecundity was detected for the estuarine copepod *Eurytemora affinis* after 10 day exposure to DEHP concentrations up to 109 ng mL^{-1}(Forget-Leray et al., 2005). In contrast, Knowles et al. (1987) reported that *Daphnia magna* reproduction was affected by DEHP exposure at concentrations ranging from 158 to 811 ng mL^{-1}. This

suggests that reproductive complications due to DEHP exposure in planktonic invertebrates are highly variable among species.

In most cases in which reproductive output changes were reported, the lower outputs were commonly attributed to altered reproductive behavior or malformed sexual organs (Gray et al., 2000; Manikkam et al., 2013; Van Wezel et al., 2000), which occurred primarily when DEHP exposure occurred during development prior to sexual maturation. As an endocrine disruptor, DEHP affects growth and reproduction, due to its effect on hormonal activity during maturation (Gray et al., 2000; Manikkam et al., 2013; Zanotelli et al., 2010). However, in this study, DEHP exposure lasted for only 5 days (one generation) and individuals matured prior to exposure, which indicates that impairment of sexual development was unlikely the reason for the observed decrease in egg production per female. It is unclear whether decreased reproductive output resulted from altered reproductive behavior in adults or from underlying biochemical effects.

4.3. Prolonged exposure - multigenerational effects

This study demonstrated that both microplastic and DEHP exposure causes significant decreases in *P. crassirostris* populations over subsequent generations. Populations were reduced by 25–40% relative to control treatments over 24 days for all exposures. Population decreases were expected under microplastic exposure. However, given the high reproductive output of *P. crassirostris,* it was speculated that the population would recover quickly after exposure ceased on day 6. Unexpectedly, populations only showed slow signs of recovery, which suggests that recovery to pre-exposure levels would take more than 18 days in the absence of microplastic. This is in contrast to the study by Cole et al. (2015) where prolonged exposure (9 days) of the marine copepod *Calanus helgolandicus* to microplastic beads did not decrease egg-production or survival. The absence of significant recovery results following microplastic exposure in the present study may be attributed to high variability among replicates.

Different DEHP exposures, whether for 6 days or 24 days, resulted in almost identical final mean population sizes after 24 days of culturing. This suggests that DEHP exposure for 6 days severely damaged the reproductive capacity of subsequent generations. Even in the absence of direct DEHP exposure (the subsequent generations were exposed through their egg/sperm-line), the reproductive capacity of subsequent generations failed to recover. Similar results were reported for rats following DEHP and other plasticizer exposures (Manikkam et al., 2013). Altered phenotypes in rats that affected sexual reproduction were epigenetically (transgenerationally) inherited by subsequent generations (Manikkam et al., 2013).

No changes in *Hsp70-like* expression were detected following microplastic or DEHP exposure. This is in stark contrast to expectations, based on post-exposure *Hsp70-like* up regulation in *Chironomus riparius* and *Chironomus tentans* when exposed to plasticizers (Lee and Choi, 2005; Morales et al., 2011). Stressors are expected to induce an increase in the expression of members of the HSP family. The upregulation of *Hsp70* is a typical stress response in order to repair cells (Morimoto, 1998). The reported increased mortality and decreased productivity of *P. crassirostris* after exposure to either treatment highlights this species is negatively impacted by microplastic and DEHP exposure. Expression of *Hsp70-like* in *P. crassirostris* was not affected, despite the individuals being clearly stressed.

H3 expression was down regulated after 6 days of exposure, but not after 18 days of recovery, following both micro PET particle and DEHP exposure. This down regulation in *H3* expression following both treatments was not transferred to offspring generations assessed on day 24. Down regulation of *H3* expression has been correlated with tumorigenesis in humans (Zhao et al., 2004, 2002). Conceivably, both DEHP and microplastics could alter cellular processes within *P. crassirostris,* resulting in the observed down regulation of *H3* expression. Further investigations may provide a better understanding of which pathways are affected and why.

While persistent population decrease over 24 days suggest an inherited effect of decreased productivity on subsequent generations, no genetic proof of epigenetic inheritance was found in the expression of *Hsp70-like* or *H3* (Youngson and Whitelaw, 2008). Further studies targeting more genes that are responsive to stressors might provide genetic proof for the ecological data of inheritance of deceased reproduction shown in this study.

4.4. Conclusion and environmental relevance

Exposure to microplastic PET particles and the plasticizer DEHP can result in severe short-term and/or long-term effects on *P. crassirostris* survival, fertility, population sizes and gene expression. *P. crassirostris* populations hint signs of recovery following exposure to microplastics for 6 days. The prolonged exposure to microplastics has severe deleterious impacts on population viability. In contrast, the exposure of *P. crassirostris* populations to DEHP resulted in persistent long-lasting effects with no indication of recovery detected for at least 18 days after exposure ceased.

Concentrations of microplastics used in this experiment are exceeding concentrations found in the environment by magnitudes. Therefore, drastic population declines of zooplankton as seen here are unlikely to occur based on the presence of microplastics alone. More concerning are the results of DEHP exposure as concentrations that affected nauplii survival and egg production of adults are well within the environmental levels. Solubility of DEHP in water is low, however, even if only present at low levels it can be readily taken up by aquatic organisms through respiration and accumulate in their tissue. Metcalf et al. (1973) exposed various aquatic organisms to DEHP dissolved in water at a concentration of 0.1 mgL^{-1} for 1–48 h. Concentrations in the body tissue (whole organisms were homogenised) ranged from 0.85 $mgkg^{-1}$ in guppy (*Lebistes reticulatus*) to 85.75 $mgkg^{-1}$ in clam (*Sphaerium striatinum*) with biomagnification factors over 24 h of 92–692, respectively. Uptake of DEHP proves to be highly variable. Clearly, further investigations regarding the impact of plasticizers on species from the base of the food web are required to better understand the environmental impact of plastic pollution.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors contributed equally.

Acknowledgment

Logistical and financial support for this project was provided by James Cook University. Preliminary plasticiser concentrations were determined by Dr. Zhiyong Xie at the Helmholtz-Zentrum Geesthacht.

Appendix A. Supporting information

Supplementary information associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.03.029.

References

Agnew, D.J., 2000. The illegal and unregulated fishery for toothfish in the Southern Ocean, and the CCAMLR catch documentation scheme. Mar. Policy 24, 361–374.
Alajmi, F., et al., 2015. Domestication as a novel approach for improving the cultivation of calanoid copepods: a case study with Parvocalanus crassirostris. PLoS One 10.
Alajmi, F., Zeng, C., 2013. Evaluation of microalgal diets for the intensive cultivation of the tropical calanoid copepod, Parvocalanus crassirostris. Aquac. Res. 46, 1025–1038.
Alajmi, F., Zeng, C., 2014. The effects of stocking density on key biological parameters influencing culture productivity of the calanoid copepod, Parvocalanus crassirostris. Aquaculture 434, 201–207.

Andersen, C., et al., 2004. Normalization of real-time quantitative RT-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250.
Anway, M.D., et al., 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308, 1466–1469.
Anway, M.D., Skinner, M.K., 2006. Epigenetic transgenerational actions of endocrine disruptors. Endocrinology 147, 43–49.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Methodol. 57, 289–300.
Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.
Chown, S.L., et al., 2012. Continent-wide risk assessment for the establishment of nonindigenous species in Antarctica. Proc. Natl. Acad. Sci. USA 109, 4938–4943.
Cole, M., et al., 2011. Microplastics as contaminants in the marine environment: a review. Mar. Pollut. Bull. 62, 2588–2597.
Cole, M., et al., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646–6655.
Cole, M., et al., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environ. Sci. Technol. 49, 1130–1137.
R Core Team, 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Dargnat, C., et al., 2009. Phthalate removal throughout wastewater treatment plant: case study of Marne Aval station (France). Sci. Total Environ. 407, 1235–1244.
Desforges, J.-P.W., et al., 2015. Ingestion of microplastics by zooplankton in the Northeast Pacific Ocean. Arch. Environ. Contam. Toxicol. 69, 320–330.
Feng, J., et al., 2014. Analysis and identification of tyrosine phosphorylated proteins in hemocytes from Fenneropenaeus chinensis (Decapoda: penaeidae) infected with white spot syndrome virus. J. Crustac. Biol. 34, 453–459.
Fischer, V., et al., 2015. Plastic pollution of the Kuril-Kamchatka Trench area (NW pacific). Deep-Sea Res. 111, 399–405.
Forget-Leray, J., et al., 2005. Impact of endocrine toxicants on survival, development, and reproduction of the estuarine copepod Eurytemora affinis (Poppe). Ecotoxicol. Environ. Saf. 60, 288–294.
Fromme, H., et al., 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water Res. 36, 1429–1438.
Galgani, F., et al., 2015. Global distribution, composition and abundance of marine litter. Marine anthropogenic litter. Springerpp. 29–56.
Gray, L.E., et al., 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol. Sci. 58, 350–365.
Horn, O., et al., 2004. Plasticizer metabolites in the environment. Water Res. 38, 3693–3698.
Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. 33, 245–254.
Kaposi, K.L., et al., 2014. Ingestion of microplastic has limited impact on a marine larva. Environ. Sci. Technol. 48, 1638–1645.
Kearse, M., et al., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.
Knowles, C.O., et al., 1987. Chronic effects of DI-2-ethylhexyl phthalate on biochemical composition, survival and reproduction of Daphnia magna. Environ. Toxicol. Chem. 6, 201–208.
Latini, G., et al., 2010. Materials degradation in PVC medical devices, DEHP leaching and neonatal outcomes. Curr. Med. Chem. 17, 2979–2989.
Laver, C.R., Taylor, J.S., 2011. RT-qPCR reveals opsin gene upregulation associated with age and sex in guppies (Poecilia reticulata)-a species with color-based sexual selection and 11 visual-opsin genes. BMC Evol. Biol. 11, 1.
Lawson, T.J., Grice, G.D., 1973. The developmental stages of Paracalanus Crassirostris Dahl, 1894 (Copepoda, Calanoida). Crustaceana 24, 43–56.
Lee, K.-W., et al., 2013. Size-dependent effects of micro polystyrene particles in the marine copepod Tigriopus japonicus. Environ. Sci. Technol. 47, 11278–11283.
Lee, S.-M., Choi, J., 2005. HSP70 and HSC70 gene expression in Chironomus Tentans (Diptera, Chironomidae) larvae exposed to various environmental pollutants: potential biomarker for environmental monitoring. Chemosphere 25, 32–37.
Manikkam, M., et al., 2013. Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. PLoS One 8.
Matz, M.V., et al., 2013. No control genes required: Bayesian analysis of qRT-PCR data. PLoS One 8.
Mauchline, J., 1998. The Biology of Calanoid Copepods: The Biology of Calanoid Copepods. Academic Press, San Diego, California, USA.
McKinnon, A., Ayukai, T., 1996. Copepod egg production and food resources in Exmouth Gulf, Western Australia. Mar. Freshw. Res. 47, 595–603.
McKinnon, A.D., et al., 2005. Mesozooplankton dynamics in nearshore waters of the Great Barrier Reef. Estuar. Coast. Shelf Sci. 63, 497–511.
McKinnon, A., Duggan, S., 2014. Community ecology of pelagic copepods in tropical coastal waters. Copepods: Diversity, Habitat and Behavior. Nova Scotia Publishers, pp. 25–49.
Metcalf, R.L., et al., 1973. Uptake and fate of Di-2-ethylhexyl phthalate in aquatic organisms and in a model ecosystem. Environ. Health Perspect. 4, 27.
Morales, M., et al., 2011. Characterization of Hsp70 gene in Chironomus riparius: expression in response to endocrine disrupting pollutants as a marker of ecotoxicological stress. Comp. Biochem. Physiol. C 153, 150–158.
Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. 12, 3788–3796.
Naito, W., et al., 2006. Screening-level risk assessment of di (2-ethylhexyl) phthalate for aquatic organisms using monitoring data in Japan. Environ. Monit. Assess. 115, 451–471.
Park, J., Gan, S.-Y., 2014. Microplastic management for preventing risk of persistent/bioaccumulative substance. J. Environ. Pol. 13, 65–98.
Peijnenburg, W.J.G.M., Struijs, J., 2006. Occurrence of phthalate esters in the environment of the Netherlands. Ecotoxicol. Environ. Saf. 63, 204–215.
Rhee, J.-S., et al., 2009. Heat shock protein (Hsp) gene responses of the intertidal copepod Tigriopus japonicus to environmental toxicants. Comp. Biochem. Physiol. C 149, 104–112.
Rochman, C.M., et al., 2014. Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. Sci. Total Environ. 493, 656–661.
Seo, J., et al., 2006. Small heat shock protein 20 gene (Hsp20) of the intertidal copepod Tigriopus japonicus as a possible biomarker for exposure to endocrine disruptors. B. Environ. Contam. Tox. 76, 566–572.
Singh, S., Li, S.S.-L., 2012. Epigenetic effects of environmental chemicals bisphenol a and phthalates. Int. J. Mol. Sci. 13, 10143–10153.
Skinner, M.K., et al., 2011. Epigenetic transgenerational actions of endocrine disruptors. Reprod. Sci. 31, 337–343.
Uren-Webster, T.M., et al., 2010. Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquat. Toxicol. 99, 360–369.
Van Wezel, A., et al., 2000. Environmental risk limits for two phthalates, with special emphasis on endocrine disruptive properties. Ecotoxicol. Environ. Saf. 46, 305–321.
Vartiainen, J., et al., 2011. Health and environmental safety aspects of friction grinding and spray drying of microfibrillated cellulose. Cellulose 18, 775–786.
Veilleux, H.D., et al., 2015. Molecular processes of transgenerational acclimation to a warming ocean. Nat. Clim. Change 5, 1074–1078.
Wagner, M., et al., 2014. Microplastics in freshwater ecosystems: what we know and what we need to know. Environ. Sci. Eur. 26, 1–9.
Weber, C.I., 1991. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency.
Wright, S.L., et al., 2013. The physical impacts of microplastics on marine organisms: a review. Environ. Pollut. 178, 483–492.
Wu, S., et al., 2010. Dynamic epigenetic changes involved in testicular toxicity induced by Di-2-(ethylhexyl) phthalate in mice. Basic Clin. Pharmacol. 106, 118–123.
Ye, T., et al., 2014. Exposure to DEHP and MEHP from hatching to adulthood causes reproductive dysfunction and endocrine disruption in marine medaka (Oryzias melastigma). Aquat. Toxicol. 146, 115–126.
Youngson, N.A., Whitelaw, E., 2008. Transgenerational epigenetic effects. Annu. Rev. Genom. Hum. Genet. 9, 233–257.
Zama, A.M., Uzumcu, M., 2010. Epigenetic effects of endocrine-disrupting chemicals on female reproduction: an ovarian perspective. Front. Neuroendocr. 31, 420–439.
Zanotelli, V.R., et al., 2010. Long-term exposure to bis (2-ethylhexyl) phthalate (DEHP) inhibits growth of guppy fish (Poecilia reticulata). J. Appl. Toxicol. 30, 29–33.
Zhao, L.-L., et al., 2009. Effects of three phthalate esters on the life-table demography of freshwater rotifer Brachionus calyciflorus Pallas. Aquat. Ecol. 43, 395–402.
Zhao, Y., et al., 2004. Down-regulation of Betaig-h3 gene is involved in the tumorigenesis in human bronchial epithelial cells induced by heavy-ion radiation. Radiat. Res. 162, 655–659.
Zhao, Y.L., et al., 2002. Downregulation of Betaig-h3 gene is causally linked to tumorigenic phenotype in asbestos treated immortalized human bronchial epithelial cells. Oncogene 21, 7471–7477.