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Toxic effects of leachates from plastic pearl-farming gear on embryo-larval development in the pearl oyster *Pinctada margaritifera*

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ABSTRACT

Pearl-farming leads to significant plastic pollution in French Polynesia (FP) as the end of life of most farming gear is currently poorly managed. Plastic debris released into the aquatic environment accumulates, with potentially detrimental effects on the lagoon ecosystem and pearl oyster *Pinctada margaritifera*, a species of ecological, commercial and social value. Here, we tested the effects of leachates from new (N) and aged (A) plastic pearl-farming gear (spat collector and synthetic rope) obtained after 24 h and 120 h incubation, on the embryo-larval development of the pearl oyster using an *in-vitro* assay. Embryos were exposed for 24 h and 48 h to a negative control (0) and the leachate from 0.1, 1, 10 and 100 g of plastic. L^{-1}. After 24 h exposure to leachate at 100 g.L^{-1}, effects were observed on embryo development (−38% to −60% of formed larvae) and mortality (+72% to +82%). Chemical analyses of plastic gear indicated the presence of 26 compounds, consisting of organic contaminants (PAHs) and additives (mainly phthalates). Screening of leachates demonstrated that these compounds leach into the surrounding seawater with an additional detection of pesticides. Higher levels of phthalates were measured in leachates obtained from new (6.7–9.1 μg.L^{-1}) than from aged (0.4–0.5 μg.L^{-1}) plastics, which could be part of the explanation of the clear difference in toxicity observed after 48 h exposure at lower concentrations (0.1–10 g.L^{-1}), associated with mortality ranging from 26 to 86% and 17–28%, respectively. Overall, this study suggests that plastic gear used in the pearl-farming industry releases significant amounts of hazardous chemicals over their lifetime, which may affect pearl oyster development that call for *in-situ* exploration.

1. Introduction

Driven by growing demand, plastics production continues to increase worldwide reaching 359 million tons in 2018 (PlasticsEurope, 2019). Ultimately, however, the final destination for anthropogenic pollutants from land or sea, including plastics, is the marine environment (Halpern et al., 2008). A predicted 4.8–12.7 million tons of plastic waste enter the ocean every year (Jambeck et al., 2015), leading to an estimated 5 trillion pieces of plastic (over 250,000 tons) floating at the sea surface (Eriksen et al., 2014). Besides sampling campaigns and monitoring efforts on plastic debris, microplastics (MPs, plastic particles < 5 mm) have recently received increasing attention. Given their ubiquitous nature and small size, MPs are likely ingested by a wide range of marine organisms leading to various biological effects, as demonstrated under laboratory conditions (e.g. Wright et al., 2013; Rochman et al., 2016; Anbumani and Kakkar, 2018). Most laboratory experiments on MP exposure emphasise the biological effects induced by ingestion but distinguishing physical effects from chemical ones remains challenging, despite an increasing body of evidence showing the role of chemical aspects of the observed toxicity (Karami, 2017; Anbumani and Kakkar, 2018). Indeed, plastic debris may also act as a vector of hazardous chemicals, such as

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Abbreviations

BaP	Benzo[a]pyrene
DBP	Dibutyl phthalate
DDT	Dichlorodiphenyltrichloroethane
DEHA	Bis(2-ethylhexyl) adipate
DEHP	Bis(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DMP	Dimethyl phthalate
EC_{50}	Half maximal effective concentration
FP	French Polynesia
GC/MS	Gas-chromatography coupled to mass spectrometry
HCH	Hexachlorocyclohexane
HDPE	High-density polyethylene
LC_{50}	Median lethal concentration
LDPE	Low-density polyethylene
MPs	Microplastics
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PC	Polycarbonate
PCBs	Polychlorinated biphenyls
PE	Polyethylene
PET	Polyethylene terephthalate
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
SLE	Supported liquid extraction
TD	Thermal desorption

additives (e.g. plasticisers, stabilisers, antioxidants, catalysts; Lithner et al., 2011; Hermabessiere et al., 2017) and/or adsorbed chemicals (e.g. PCBs, PAHs, pesticides), which can leach into the surrounding environment and be transferred into marine organisms (Mato et al., 2001; Teuten et al., 2009). Although the transfer of adsorbed pollutants by plastic may be negligible compared with other natural pathways at sea (e.g. detritus, colloids, bacteria, phytoplankton, organic matter, food, etc.), this is supposedly not the case for plastic additives (Koelmans et al., 2016). Several studies have demonstrated enhanced desorption of chemicals in the gut upon plastic ingestion, resulting in their bioaccumulation and the deterioration of key physiological functions of the organisms (e.g. Browne et al., 2013; Koelmans et al., 2013, 2014; Rochman et al., 2013, 2014). Most research carried out on toxic effects mediated by plastics has been conducted by direct exposure of organisms to contaminated plastic debris once ingested. Nevertheless, few studies have demonstrated the indirect toxicity mediated by plastic leachates, i.e. desorption of chemicals into the surrounding environment in the absence of plastic ingestion.

The first leaching studies on marine organisms were conducted on plastic consumer products (e.g. DVD-case, biodegradable bag, phone cover, plastic cup, etc.) of various polymer types (Lithner et al., 2009, 2012; Bejgarn et al., 2015). The acute toxicity of non-weathered plastic leachates (including 11 plastic products) was demonstrated by significant mortality (EC_{50}) in *Daphnia magna* occurring after 48 h exposure to concentrations ranging from 2 to 235 $g.L^{-1}$ (Lithner et al., 2012). Similarly, exposure of the copepod *Nitocra spinipes* to leachates of non-weathered products at 100 $g.L^{-1}$ for 96 h led to LC_{50} from 7% to 100% leachate (Bejgarn et al., 2015). After weathering, toxicity either increased or decreased depending on the plastic type (Bejgarn et al., 2015). Exposure to plastic leachates obtained from seven polymer groups (HDPE, LDPE, PP, PVC, PC, PET and PS) also increased barnacle nauplii mortality (+11% to +22%) at the highest tested concentration (0.5 m^2 plastic materials. L^{-1}) and inhibited larval settlement after 24 h from −30% to −42% (H.-X. Li et al., 2016a,b). Gandara e Silva et al. (2016) also showed a higher toxicity of leachates obtained from beached pellets (42% PE and 58% unknown composition) than from virgin pellets (PP) on embryo development of the brown mussel, with 100% and 23%, respectively, of dead or abnormal embryos, suggesting a difference in the chemical load depending on pellet history (i.e. adsorbed contaminants for beached pellets *vs.* additives for virgin pellets). Reports have underlined the importance to consider the diversity of chemicals used during plastic manufacturing and those adsorbed once plastics enter the environment, all of which could play a role in toxicity.

The level of fishing and aquaculture activity is known to contribute significantly to the amount of plastic waste discharged into the ocean (Hinojosa and Thiel, 2009; W. C. Li et al., 2016a). In French Polynesia (FP), pearl oyster aquaculture is a specific source of plastics (Andréfouët et al., 2014). Indeed, the economic decline of pearl-farming in the 2000s left behind it the vestiges of intense activity as many concessions later closed and the associated rearing structures were abandoned. Added to this a common practice on operational pearl farms is to dispose of inoperative farming structures by scuttling them at the bottom of lagoons. The inventory carried out by Andréfouët et al. (2014) in the lagoon of Ahe (FP) where pearl oysters are cultured revealed large quantities of collectors, ropes, buoys and nylon ties. All of these rearing structures, essentially made of synthetic materials, are accumulating over time in lagoons and may fragment and weather. This situation is worsened by the semi-enclosed environments of some of these lagoons, which could favour MP accumulation (Andréfouët et al., 2014). A recent laboratory experiment conducted by Gardon et al. (2018) highlighted the impact of commercial virgin micro-PS (polystyrene microbeads) on energy balance and gametogenesis of the pearl oyster *Pinctada margaritifera*, and pointed out the need to differentiate physical and chemical effects so as to better understand the risk. In order to go further, estimating the effects of local scenario for coastal organisms will be necessary to assess risks, here associated with the presence and ageing of pearl-farming structures in Polynesian lagoons. Experimental work is a first step which opens up questions on the field to ultimately alert and support decision.

In this study, we conducted experiments to evaluate the toxicity of leachates obtained from new and aged plastic farming gear on embryo-larval development of the pearl oyster (*P. margaritifera*). Focussing on non-ingestion pathways and therefore the effects mediated through chemicals leaching into the water, embryos of *P. margaritifera* were exposed to leachates from spat collectors and synthetic rope: two types of farming gear that oysters encounter in the field. In this context, we performed large-scale chemical screening on the plastic gear and plastic leachates used for the toxicological assay in order to identify the major contaminants in fractions of both new and aged gear.

2. Materials and methods

2.1. Plastic selection

Based on Andréfouët et al. (2014), our study focused on the most abundant plastic debris collected in FP pearl-farming atoll lagoons, specifically spat collectors (shade-mesh) and synthetic ropes mostly made from polypropylene (PP) and polyethylene (PE),

respectively, polymers that represent more than 50% of global plastic production (PlasticEurope, 2019). New plastic gear was obtained from local suppliers in FP (POE import) and aged ones were collected directly in the vicinity of a pearl-farm located in Manihi (14°24′10″S, 145°57′29″W, FP). FTIR microspectroscopy analysis confirmed that the spat collectors and synthetic rope used in this study consisted of PP and PE, respectively, for both the new and aged gear; no additional features were identified in the spectra of any samples (Fig. S1).

2.2. Microplastic production

Aged spat collectors and synthetic ropes were washed in saline water (100 g of NaCl.L^{-1}) aiming to remove most of the microorganisms potentially present, then rinsed thoroughly and dried in a proofer at 60 °C for 48 h. No pre-washing of new equipment was done so as to test the first leaching water. To facilitate leaching and increase the surface area available during incubation, spat collectors (MPs-1) and synthetic ropes (MPs-2) coming from both new and aged plastic gear were cut with clean, stainless steel scissors into $\leq$ 5 mm pieces. Pictures were taken of the MPs produced using an illuminated binocular magnifier (Leica M80) equipped with a camera (Leica MC170 HD) (Fig. 1).

2.3. Leachate preparation

Leaching was performed according to the European Committee for Standardization CEN/TC 444 – EN 12457–4 (CEN, 2002) increasing leaching time from 24 h in the norm to 120 h. These leaching times were chosen in order to cover the range of leaching time tested in the literature (i.e. mainly 24 h and up to 96 h; Lithner et al., 2012; Bejgarn et al., 2015; Nobre et al., 2015; Gandara e Silva et al., 2016; Li et al., 2016a) and exceed this time through the 120 h-leachate to assess toxicity over a longer leaching period.

Leachates were prepared by putting 50 g of MPs (25 g MPs-1 and 25 g MPs-2) in 500 ml seawater in a previously autoclaved 1-L glass bottle (100 g.L^{-1}). The seawater used for preparing the leachates was pumped from the lagoon of Vairao (17°48′23″S, 149°2′41″W, Tahiti, FP), mechanically filtered on a 1 µm sock filter, UV treated and autoclaved (salinity = 35 psµ; pH = 8.2). Leaching was performed for 24 h and 120 h under artificial light (400–750 nm, 30 W, 2400–2700 lm) at 28 °C on a magnetic agitator at 600 rotations per minute (rpm). Five leaching treatments were applied: new MPs 24 h (N-24) and 120 h (N-120) leachates, aged MPs 24 h (A-24) and 120 h (A-120) leachates and a control seawater treatment with no MPs. After the leaching period, leachates were separated from MPs by filtration on GF/C filters (1.2 µm of porosity, Ø 90 mm, Whatman™) and filtered a second time on 0.22 µm Millipore filters (Whatman™) in 500 ml glass bottles previously autoclaved (similarly for the control). For each treatment, 100 ml of pure leachate (100 g.L^{-1}) were sampled for chemical analyses. A diagram of the experimental design is given in Fig. 2.

2.4. Broodstock conditioning and spawning

Adult *Pinctada margaritifera* individuals (aged 8–10 years) were collected from Vairao lagoon (Ifremer marine concession No. 8120/MLD: 17°48′26.0″S, 149°18′14.4″W, Tahiti, FP). The animals were cleaned and transferred to the laboratory to serve as genitors for the embryo-larval exposure. For this toxicity test, 40 females and 30 males were placed in seawater at 21 °C with aeration and left in the dark overnight in an air-conditioned room. Following this period, the cold-water bath was drained and replaced with filtered seawater at 28 °C. Gamete release was stimulated by thermal shock (Le Moullac et al., 2003). Individuals that responded to this thermal shock were each isolated in a 2-L glass beaker with 1-µm-filtered and UV-treated seawater. One female and one male were selected for the experiment. Oocyte and sperm concentrations were determined using a Multisizer™ 3 Coulter Counter (Beckman Coulter, Inc., Brea, CA) equipped with a 100 µm aperture tube. Gametes were pooled into a 2-L glass beaker at a sperm/oocyte ratio of 100:1 (Hui et al., 2011) to allow fertilisation (90% fertilisation success) before the beginning of the exposure.

2.5. Chemical analyses

2.5.1. Screening of plastics

To allow the analysis of a maximum number of compounds, two protocols were employed for each plastic sample (new and aged) used to produce the leachates. The first protocol carried out a thermal desorption (TD) directly on plastics and analysed chemical compounds by gas-chromatography coupled to mass spectrometry (GC/MS). The second protocol consisted of dissolving plastics in a solvent (supported liquid extraction [SLE]) and analysed compounds after liquid injection (GC/MS).

Quantification by TD-GC/MS. Approximately 1 mg of each plastic

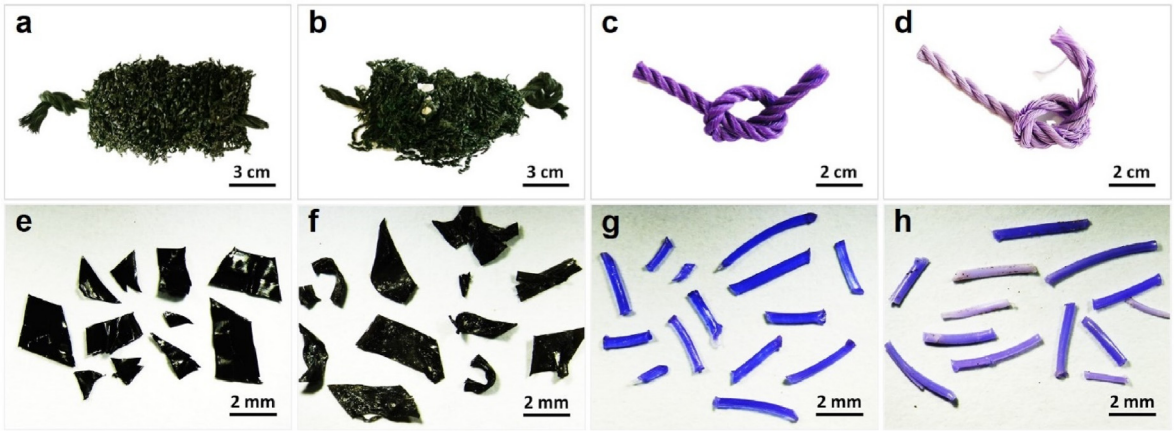

Fig. 1. Plastic pearl-farming gear (a–d) used to prepare microplastics (e–h) employed for leachate preparation. Pictures show new (a, e) and aged (b, f) spat collectors, and new (c, g) and aged (d, h) synthetic rope in whole form and cut into microplastics.

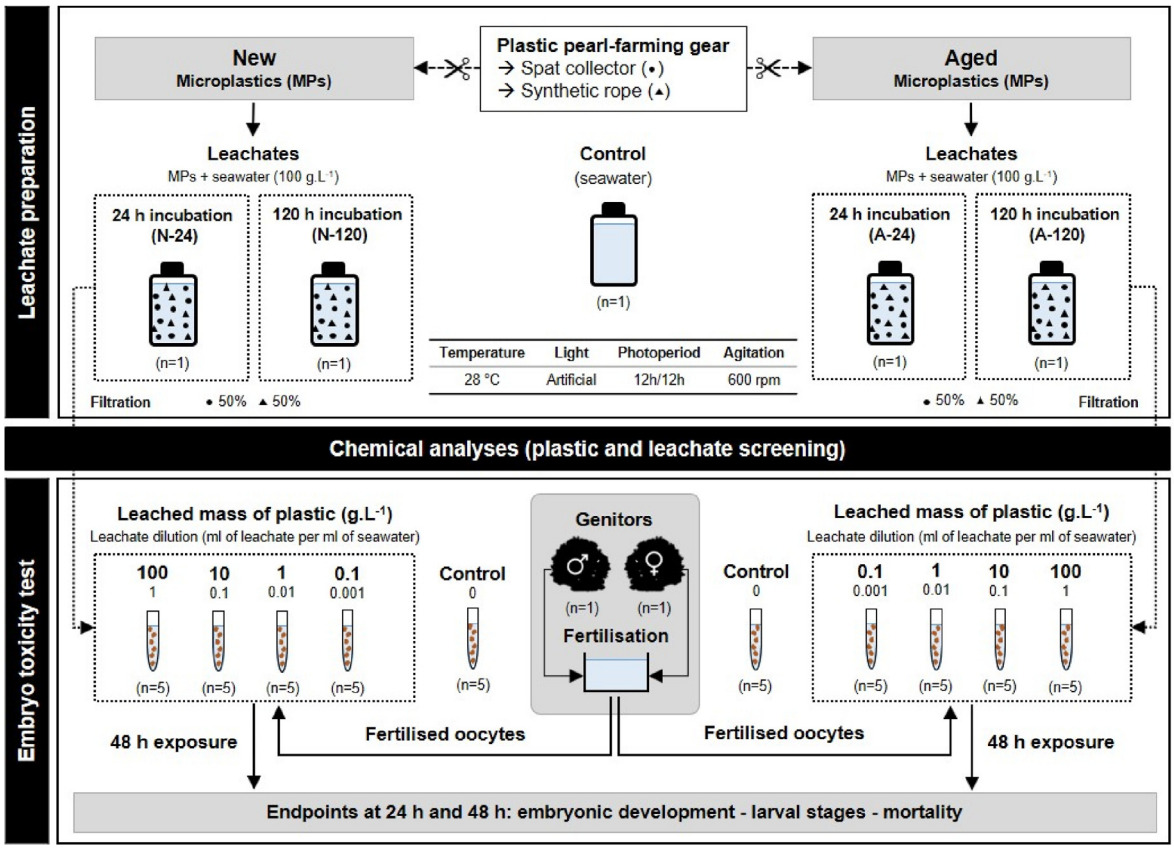

Fig. 2. Experimental design.

type was weighed with precision then transferred to a pyrolysis vial. GC/MS analysis was performed with a gas chromatograph (Agilent 7890N) equipped with a Combipal MPS2 multifunctional injection system (Gerstel, Switzerland), used in *'splitless'* mode, and a pyrolyser (Gerstel Pyro). The temperature-controlled cooling injection system (CIS) was programmed from −35 °C (0.05 min) to 340 °C (4 min) at 12 °C.s-1. During desorption time, the temperature of the interface was maintained at 350 °C. The oven temperature program was from 50 °C (0.5 min) to 150 °C at 15 °C.min-1, then up to 315 °C (5 min) at 7 °C.min-1. The carrier gas was helium at a constant rate of 1.2 ml. min-1. The capillary column was an HP-5-MS (Agilent Technologies): 30 m × 0.25 mm ID × 0.25 μm (film thickness). The chromatograph was coupled to a 5975N mass spectrometry detector. Semi-quantitative compounds analysis was carried out in single ion monitoring (SIM) mode with the molecular ion of each compound of interest (minimum of 1.5 cycles. s-1) (listed in Tables S1 and S2). Limits of quantification (LOQ) were calculated according to Shrivastava and Gupta (2011) using the calibration curves method. Analytes were quantified relatively to deuterated compounds using a calibration curve ranging from 0.01 ng to 10 ng. For PAHs, PCBs and pesticides quantification, naphthalene-d8, biphenyl-d10, phenanthrene-d10, pyrene-d10, chrysene-d12, benzo(a)pyrene-d12 and benzo(g, h, i)perylene-d12 were used as standards. For plastic additives, phthalates and PBDE, di(2-ethylhexyl) phthalate (DEHP) d4 and BDE 77 were respectively used as standards. All standards were obtained from LGC Standard (Wesel, Germany) and Interchim (Montluçon, France).

Dosage by SLE-GC/MS. Approximately 50 mg of each plastic type were dissolved in 10 ml of toluene (reflux during 10 min) then reconcentrated to 250 μl. A volume of 10 μl of the solution was doped with deuterated PAHs, deuterated phthalates and PBDEs, then analysed by GC/MS in the same conditions as previously described for TD-GC/MS.

2.5.2. Screening of leachates

A stir bar sorptive extraction was performed before TD-GC/MS analysis as decribed by Lacroix et al. (2014). At first, a polydimethylsiloxane stir bar (Twister 20 mm × 0.5 mm, Gerstel, Mülheim an der Ruhr, Germany) was placed in the 100 ml leachate (opaque glass flask) then closed and placed on a magnetic agitator (MIX15, Munich, Germany) for 16 h of extraction at 700 rpm in the dark at room temperature. After the extraction step, stir-bars were retrieved, rinsed with distilled water and placed on a gas chromatography system Agilent 7890A coupled with an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies, LittleFalls, USA) and equipped with a thermal desorption unit combined with a cooled injection system (CIS) (Gerstel, Mülheim an der Ruhr, Germany). Thermodesorption was performed at 280 °C for 6 min and samples where then cryofocused in the CIS at −10 °C. Injection in the GC-MS/MS system was carried out in splitless mode and the CIS was heated to 300 °C at 12 °C.s-1. The GC temperature program was set as follows: 70 °C for 0.5 min, then increase to 150 °C at 20 °C.min-1 and finally increase to 300 °C at 7 °C.min-1, maintained for 5 min. A Rxi-5MS (30 m, 0.25 mm, 25 mm thickness) (Restek, Lisses, France) capillary column was used. Helium was used as a carrier gas with a constant flow rate of 1 ml. min-1. The quantitative analysis of PAHs, PCBs, pesticides, PBDEs and phthalates was carried out by internal calibration in multiple reaction monitoring mode with two transitions for each compound, one (Quantify) for quantification, the other (Qualify) for confirmation of the molecular nature by calculating the ratio Qualify/Quantify and by comparison with reference values of pure

compounds. The acquisition frequency for each fragment was 2 cycles. s-1.

2.6. Toxicity test on pearl oyster embryos

The ecotoxicological assay consisted of a factorial design with two factors: treatment (i.e. control, N-24, A-24, N-120 and A-120 leachates) and leachate concentration; 1 (pure leachates made from 100 g of plastic. L^{-1}), 0.1, 0.01 and 0.001 ml of leachates per ml of seawater ($ml.ml^{-1}$), and a control treatment (seawater with no addition of leachates). Dilutions were realised by mixing leachates with filtered seawater (UV-treated and filtered on a 0.22 μm Millipore filter). The leachate concentrations can be expressed as leached mass of plastic. L^{-1}. Applying this methodology, the resulting exposure levels used throughout the present study were: 0 (control), 0.1, 1, 10 and 100 $g.L^{-1}$. Even though the presence of plastic products with the leachate seems to be an important factor when assessing plastic toxicity (Nobre et al., 2015; Gandara e Silva et al., 2016), we used leachate alone in order to focus on chemical toxicity and avoid confounding factors in this experiment (Lithner et al., 2009, 2012; Bejgarn et al., 2015). An embryo toxicity test was performed according to the ASTM guideline E724-98(2012) (ASTM, 2012) and implemented to our model species *P. margaritifera* (black-lip pearl oyster).

The toxicity test was conducted in five replicates in glass test tubes (18 × 150 mm) previously annealed at 450 °C. Fertilised oocytes from the fertilisation were filtered on a 20 μm mesh sieve and concentrated in a glass beaker to obtain a final concentration of 7800 fertilised oocytes. ml^{-1}. We added 300 fertilised oocytes to each test tube, thus starting the exposure period of the toxicity test. Population monitoring was established after 24 h and 48 h exposure by homogenising the medium, extracting 4 ml and adding 2–3 drops of formalin. Population analysis was performed using an optical microscope (ZEISS Axiostar) for the total count (magnification × 200) and population characterisation (magnification × 400). We determined the percentage of embryonic development, defined here as the total count of formed larvae (i.e. trocophore larvae, and both alive and dead D-stage larvae) out of 300 fertilised oocytes at 24 h and associated percentages of larval stages (i.e. trocophore, D-stage and dead larvae) at 24 h and 48 h, respectively, in order to obtain population proportions and cumulative mortality (developmental arrest and dead larvae) (ASTM, 2012). Dead larvae were discriminated from D-stage larvae by their empty shells.

2.7. Statistical analysis

All data were expressed as percentages (mean ± standard deviation) and transformed by the arcsine square-root function before statistical analysis. Normality and homoscedasticity were tested with Shapiro–Wilk and Levene's tests, respectively. Data complying with the conditions of normal distribution and homogeneity of variance were analysed using a two-way parametric ANOVA to test the differences in variables between factors (i.e. treatment and leachate concentration). Whenever necessary, a Tukey's post-hoc test was used to determine the significant differences between the averages of each group. When assumptions of normality and homogeneity of variance were not met, we used the two-way non-parametric Scheirer–Ray–Hare (SRH) ANOVA with interaction. To avoid ranking comparison, Dunn's test was used on pooled factors in order to determine the significant differences between the averages of each group. Data presented in the figures are not square root transformed. In all cases, $p < 0.05$ was defined as the threshold of statistically significant differences. All analyses were performed in statistics software RStudio v3.5.

3. Results

3.1. Screening of plastics and leachates

3.1.1. Chemicals in plastic gear

Among all targeted PAHs, exploratory chemical analyses identified the presence of 20 PAHs (Fig. S2). Relative abundances were noticeably similar between the two extraction methods, with complementary information depending on the compounds. Corresponding raw data are given in Table S1. Regarding additives, phthalates (DMP, DEP, DBP, BBP, DEHA and DEHP) and the antioxidant Irgafos 168® were detected with both protocols. However, the SLE-GC/MS method, which integrates a dissolution phase in solvent, revealed outliers in the experimental blank linked to a phthalate contamination. This kind of contamination is well known in chemical laboratories and seawater used for calibration curves (i.e. no water is “phthalate free”; Fankhauser-Noti and Grob, 2007), making it particularly difficult to determine phthalates in environmental matrices (e.g. tissue, water or sediment). In contrast, the TD-GC/MS method provided more relevant values with lower contamination; results are presented in Figure S3 and the corresponding raw data are given in Table S2. After blank correction (i.e. samples were adjusted by substracting the contribution from the blank control), phthalate concentrations reached 79 and 73 $\mu g\,g^{-1}$ for new and aged spat collectors, respectively, and, 252 and 78 μg.g-1 for new and aged synthetic ropes, respectively. Screening was also performed for PCBs, pesticides and PBDEs and no compounds of these three families were detected in spat collectors and synthetic ropes.

3.1.2. Chemicals in leachates

Chemical analyses performed on leachates quantified total concentrations of PAHs, PCBs, pesticides, PBDEs and phthalates for each treatment (Fig. 3). PAH concentrations were 10.8, 4.7, 5.4 and 7.9 $ng.L^{-1}$ in N-24, N-120, A-24 and A-120, respectively, and 6.4 $ng.L^{-1}$ in the control treatment. PCBs, at a concentration of 2.2 $ng.L^{-1}$, were only found in the N-24 treatment. Concerning pesticides, concentrations of 7.5, 6.0, 7.3 and 40.6 $ng.L^{-1}$ were found in N-24, N-120, A-24 and A-120, respectively, and 21.7 $ng.L^{-1}$ in the control treatment. PBDEs were detected in the control treatment only at a concentration of 2.4 $ng.L^{-1}$. Phthalate concentrations reached 6,681, 9,077, 539 and 430 $ng.L^{-1}$ for N-24, N-120, A-24 and A-120, respectively, and 2314 $ng.L^{-1}$ in the control treatment. Detailed results are given in Table S3.

3.2. Embryo-larval development

3.2.1. Mortality

After 24 h exposure, the two-way ANOVA on cumulative mortality (Table S4) indicated significant effects of treatment ($p = 0.0031$) and leachate concentration ($p < 0.001$). Pairwise comparisons showed a significantly higher cumulative mortality in N-24, A-24, N-120 (100%) and A-120 (90.1 ± 3.0%) at 100 $g.L^{-1}$ ($p < 0.001$) compared with the control (18.3 ± 11.1%) (Fig. 4a). After 48 h exposure, the two-way ANOVA on mortality (Table S4) revealed a highly significant effect of treatment ($p < 0.001$), leachate concentration ($p < 0.001$) and a small interaction ($p = 0.0211$). Pairwise comparisons showed significant differences in cumulative mortality for control (10.0 ± 13.8%) *vs.* N-24, A-24, N-120 (100%) and A-120 (93.8 ± 4.6%) at 100 $g.L^{-1}$ ($p < 0.001$); *vs.* N-24 at 10 $g.L^{-1}$ (58.8 ± 30.8%, $p = 0.0064$); and *vs.* N-120 at 0.1 and 1 $g.L^{-1}$ (85.9 ± 3.0% and 65.7 ± 24.5%, respectively, $p < 0.001$) and 10 $g.L^{-1}$ (59.5 ± 19.9%, $p = 0.0034$) (Fig. 4b). Detailed Tukey's test results on mortality data at 24 h and 48 h, including the significant differences between other treatments, are given in Table S5.

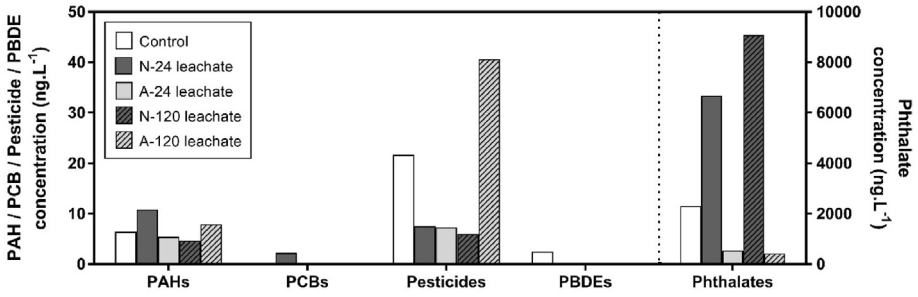

Fig. 3. Chemical compounds quantified by SBSE-TD-GC/MS in pure 24 h and 120 h leachates from new and aged plastics at 100 g.L^{-1}. PAH: polycyclic aromatic hydrocarbons, PCB: polychlorinated biphenyls, PBDE: polybrominated diphenyl ethers.

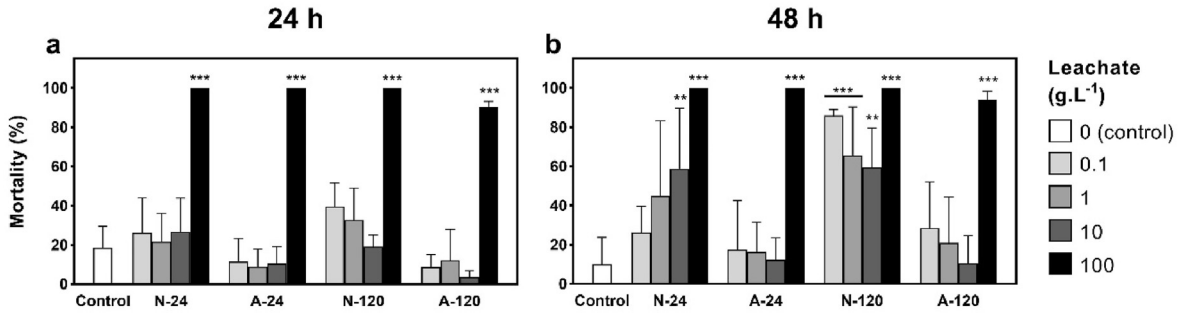

Fig. 4. Mortality after 24 h (a) and 48 h (b) exposure to leachates from new and aged plastic gear. Mean (n = 5) ± SD. Asterisks indicate statistically significant differences ($p < 0.05$) from the control (Tukey's post-hoc test: " ** ", $p \leq 0.01$; " *** ", $p \leq 0.001$).

3.2.2. Embryonic development

Embryo development (Table S6) was significantly affected by treatment (ANOVA, $p = 0.0011$) and leachate concentration (ANOVA, $p < 0.001$). Indeed, pairwise comparison showed a significant alteration of embryo development in the number of larvae formation from fertilised oocyte in N-24 (44.7 ± 6.8%, $p = 0.0041$), A-24, N-120 (30.3 ± 14.9% and 22.4 ± 2.9%, respectively, $p < 0.001$), and A-120 (44.9 ± 12.4%, $p = 0.0043$) at the highest concentration of 100 g.L^{-1} compared with the control (82.9 ± 11.3%) (Fig. 5). Detailed Tukey's test results are given in Table S7.

3.2.3. Larval stages

At 24 h, the two-way ANOVA performed on the percentage of trocophore larvae (Table S8) revealed a significant effect of leachate concentration ($p < 0.001$) with a strong interaction with treatment ($p < 0.001$). The exposure at 100 g.L^{-1} in A-24 and N-120 led to a 100% developmental arrest at the trocophore stage except for N-24 and A-120 with 99.6 ± 0.8% and 61.7 ± 9.5%, respectively, leading to a significant difference with the control treatment (1.4 ± 1.1%, $p < 0.001$) (Fig. 6d). As a consequence of this developmental arrest, the percentage of D-stage larvae formation (Table S8) in N-24, A-24, N-120 (0.0 ± 0.0%) and A-120 (22.9 ± 7.3%) at 100 g.L^{-1} was almost null and significantly lower than the control ($p < 0.001$) (Fig. 6e). The two-way ANOVA revealed a significant effect of leachate concentration ($p < 0.001$) with a strong interaction with treatment ($p < 0.001$). Proportions of dead larvae (Table S8) were analysed by SRH test, which revealed significant effects of treatment ($p < 0.001$) and the interaction ($p = 0.0127$). The pairwise comparison revealed a significantly higher percentage of dead larvae in A-120 at 100 g.L^{-1} (15.4 ± 9.2%, $p = 0.0008$) compared with the control (0.1 ± 0.3%) (Fig. 6f). No difference with N-24, A-24 and N-120 at 100 g.L^{-1} was observed since D-stage larvae formation did not occur in these treatments and therefore none associated dead larvae. Detailed post-hoc test results performed on larval stages at 24 h are given in Table S9.

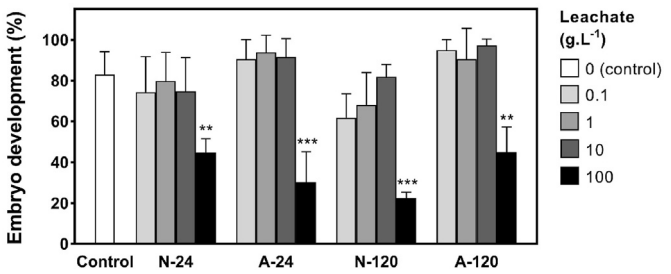

Fig. 5. Percentage of embryonic development of fertilised oocytes after 24 h exposure to leachates from new and aged plastic gear. Mean (n = 5) ± SD. Asterisks indicate statistically significant differences ($p < 0.05$) from the control (Tukey's post-hoc test: " ** ", $p \leq 0.01$; " *** ", $p \leq 0.001$).

At 48 h, similar effects were observed on larval stages (Table S10) for all leachate treatments at the highest concentration (100 g.L^{-1}) (Fig. 6g–i). In addition, effects at the lower concentrations (0.1, 1 and 10 g.L^{-1}) were observed. Pairwise comparison showed a significant decrease of D-stage larvae in N-120 at 0.1 g.L^{-1} (24.3 ± 11.4%, $p = 0.0052$) compared with the control (99.3 ± 0.6%) (Fig. 6h). Significantly higher proportions of dead larvae were also observed in N-120 at 0.1 (75.7 ± 11.4%) and 1 (53.7 ± 26.2%) g.L^{-1} ($p = 0.0021$ and $p = 0.0424$, respectively) compared with the control (0.7 ± 0.6%) (Fig. 6i). Detailed post-hoc test results performed on larval stages at 48 h are given in Table S11.

4. Discussion

4.1. Phthalates desorption from plastic gear

Leachate screening provided information on the chemicals

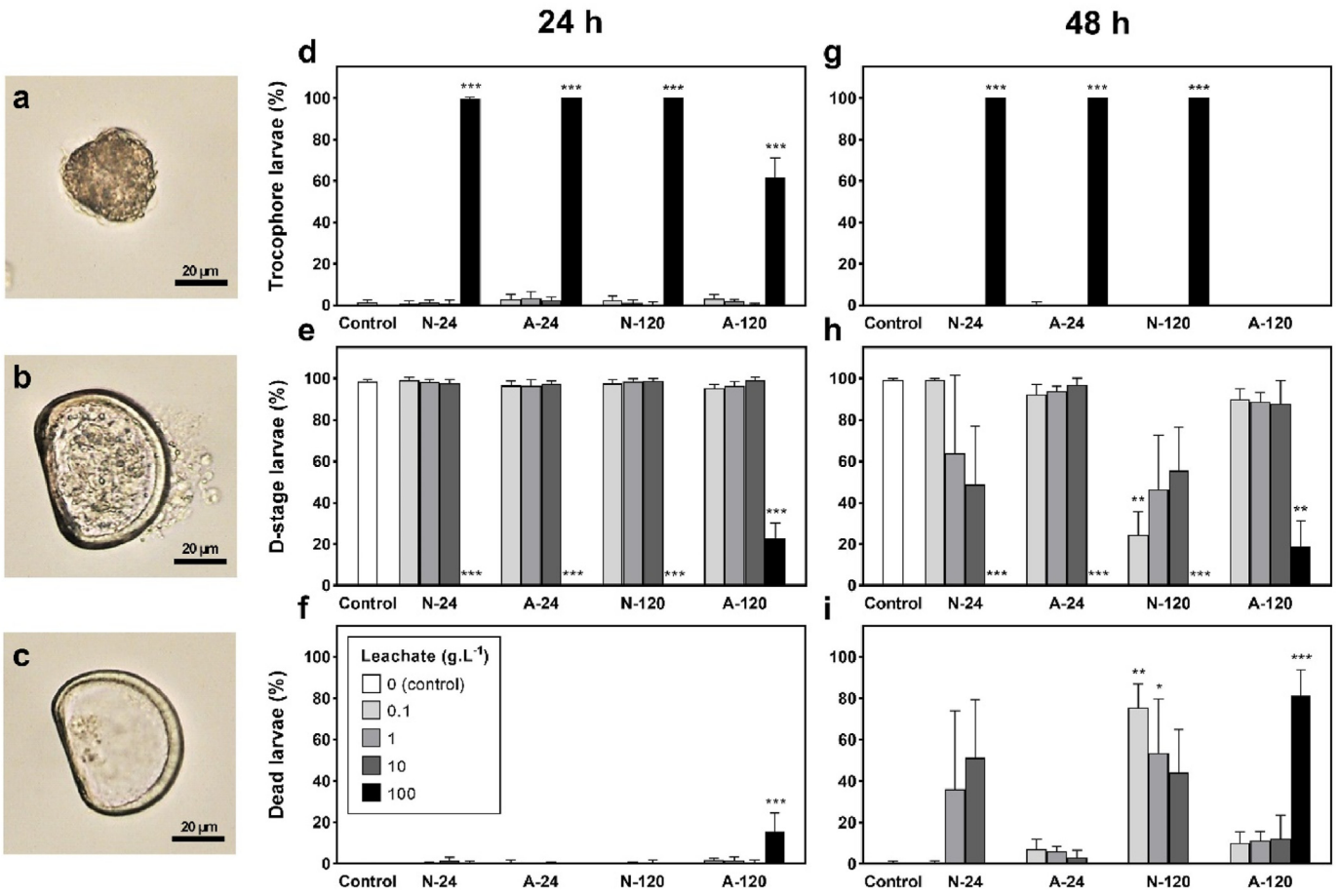

Fig. 6. Larval stages after 24 h (d–f) and 48 h (g–i) exposure to leachates from new and aged plastic gear. Pictures show the different larval stages/states: trocophore (a), D-stage (b) and dead (c) larvae. Mean (n = 5) ± SD. Asterisks indicate statistically significant differences ($p < 0.05$) from the control (" * ", $p < 0.05$; " ** ", $p \leq 0.01$; " *** ", $p \leq 0.001$).

associated with plastic collector and rope that desorbed into the surrounding media. Although some chemicals identified on these plastic samples appeared below the detection and/or quantification limit, PAHs and additives were successfully quantified in leachates in addition to the detection and quantification of PCBs, pesticides and PBDEs. Levels of PAHs, pesticides, PBDEs and phthalates quantified in the control (with no plastic gear added) indicated an existing contamination of seawater employed in this experiment and/or during sample preparation and chemical analyses (laboratory plastic). This result underline the difficulties of studying unknown and most of the time unidentifiable chemical composition of plastic leachates (Zimmermann et al., 2019). Indeed, it is well recognised that minimizing external contamination by ubiquitous plasticizers and other chemicals is a real challenge in biomonitoring studies (Ye et al., 2013). Composition of natural seawater at global scale is subject to an irreversible modification by a multitude of organic pollutants at ultra-trace levels (Dachs and Méjanelle, 2010). Using quality control blanks or samples to assess potential contamination during analysis and the accuracy of measurements is difficult since their contamination is a *quasi*-systematic phenomenon (Taylor, 1981; Fankhauser-Noti and Grob, 2007). Besides anthropogenic organic chemicals introduced in the marine environment (Dachs and Méjanelle, 2010), several reports have investigated and pointed out potential sources of contamination such as indoor air (Alcock et al., 1994; Fankhauser-Noti and Grob, 2007), solvents (including water), reagents, experimental apparatus and protection equipment for sample preparation in laboratory environment (e.g. gloves) (Sjödin et al., 2004; Ye et al., 2013). Thus, because contamination affecting quality control samples cannot be avoided completely, the concentration of samples should be adjusted by substracting the contribution from the quality control (Ye et al., 2013) as we did here. Nonetheless, we note that, for some molecules, the contamination should be present at least at the same concentration or above in the leachate treatments. However, the leachate results showed lower levels of pesticides (except for A-120), PBDEs and phthalates (only for A-24 and A-120) than the control, suggesting that we cannot exclude a potential adsorption of these chemicals by MPs (Lee et al., 2014) used for leachate preparation. Despite control contamination, a real difference in chemical composition of new and aged plastic leachates was detected concerning PAHs and pesticides families. PAHs such as benzo[a]pyrene (BaP), chrysene and pesticides as σ- and γ-hexachlorocyclohexane (HCH), chlorpyrifos and σ-endosulfan were quantified in higher levels (from 2 to 20 times higher) in the A-120 treatment, indicating a potential adsorption of these chemicals in the marine environment (Teuten et al., 2009; Lee et al., 2014). Furthermore, additives, especially phthalates, represented the highest levels of quantified chemicals in leachates, with a major part attributable to DMP and a lesser part to DEP. However, their concentration levels are under-estimated since the total phthalate concentration does not consider DBP, DEHA and DEHP (i.e. the most abundant phthalates in plastic samples) because they are ubiquitous laboratory contaminants (Fankhauser-Noti and Grob, 2007). Comparing the concentrations of phthalates between leachates,

about 12–21 times more phthalates were leached from new than from aged plastic gear after 24 h (6681 and 539 ng.L-1, respectively) and 120 h (9077 and 430 ng.L-1, respectively) incubation. Regarding the results of plastic screenings, phthalates were twice as abundant in new than in aged synthetic rope (282 and 108 μg.g-1, respectively), making a distinction between new and aged spat collectors (109 and 103 μg.g-1, respectively), suggesting a higher propensity for synthetic rope to leach into the surrounding environment. Similarly, the higher load of some PAHs and additives (DBP, DEHA and DEHP, Irgafos 168®) in the new synthetic rope compared with the aged one suggest a potential release of these compounds into seawater during their use in pearl-farming lagoons (Hermabessiere et al., 2017). This trend is less obvious concerning spat collector. If we consider the difference in total additive concentration between new and aged farming gear as relative to what is leached into the surrounding environment, we can consider an additive leaching of 6.3 μg.g-1 and 184 μg.g-1 from the spat collector and synthetic rope, respectively. This difference in leaching concentration highlights a difference in leaching properties of additives between the two main types of plastic used in pearl-farming, which is about 30 times greater from the synthetic rope (PE) than from the spat collector (PP). This divergence could be linked to the nature of polymer (H.-X. Li et al., 2016b) and to the types and concentrations of additives themselves varying between polymers and between plastic gears.

Further information on leaching properties was obtained from the assessment of leachates over time. Depending on the chemical compound, we generally quantified higher levels in leachates after 120 h than after 24 h incubation. For example, results obtained on new leachates revealed a DMP concentration from 5000 to 7500 ng.L-1 in N-24 and N-120, respectively. Similarly, γ-HCH concentration in leachates from aged plastics varied from 2.2 to 29.7 ng.L-1 in A-24 and A-120, respectively. For sorption of chemicals onto plastic, an increase over time was observed until equilibrium was reached at 24–48 h for some organic pollutants absorbed by PE floating in seawater (Bakir et al., 2014a). Other studies determined a longer period (from 200 h until more than 20 days) to reach equilibration for organic pollutants being absorbed by PE and PP (Karapanagioti and Klontza, 2008; Romera-Castillo et al., 2018). In our study, increasing chemical concentrations according to the leaching time of plastic suggest an enhanced desorption specific to compounds and physico-chemical conditions (Bakir et al., 2014b). It would be interesting to conduct further experiments to assess the concentration of chemicals over a longer period of time (weeks and months) and obtain a standard range of desorption of these gears.

Our chemical analyses support the complexity of studying the chemical aspects of plastic debris. Indeed, the composition of plastic products is generally unknown since polymer manufacturers are not required to disclose the ingredients of their products (Bolgar et al., 2015). It is also challenging to determine an exhaustive plastic composition by current analytical methodologies to assess their presence and amounts within plastics and associated leachates (Bolgar et al., 2015). Our work provides the first information about the chemical compounds associated with plastic gear used in pearl-farming, with 26 compounds identified as PAHs and additives, of which eight were leached, particularly phthalates. As with many plastic additives, phthalates are not chemically bonded to the polymer matrix and are, hence, easily dissolved in water (Clara et al., 2010). Once released from the polymer matrix, phthalates and other identified chemicals may become available for uptake by living organisms and could cause a range of effects, such as endocrine disruption that may cause adverse reproductive and developmental effects (Teuten et al., 2009; Hermabessiere et al., 2017).

4.2. Plastic leachates impair embryo-larval development in the pearl oyster

Given these chemical analyses, it is likely that the observed toxicity may be a result of the interaction of contaminants present in a complex mixture of chemicals leached from plastic gear compared to the control (Teuten et al., 2009; Engler, 2012). The high percentage of mortality (90–100%) occurring in embryos after 24 h exposure to leachates from new and aged farming gear at 100 g.L^{-1} suggests a chemical toxicity of desorbed compounds. Embryo mortality was observed with the exposure of brown mussel embryos to leachate from beached pellets (0.2 ml of pellets. ml^{-1}) up to 100% dead or abnormal embryos (Gandara e Silva et al., 2016). In our data, significant sub-lethal effects appeared at lowest concentrations after 48 h exposure to N-24 (59% at 10 g.L^{-1}) and N-120 (60–86% at all leachate concentrations), highlighting a higher toxicity from N-120 and more broadly from new plastic gear. This difference in toxicity between leachates from new and aged plastic could be discussed regards to the difference in phthalate concentrations (+6142 and + 8647 ng.L-1 in new plastic leachates after 24 h and 120 h incubation, respectively). Some studies that assessed the toxicity of leachates from new plastic materials to aquatic organisms (Lithner et al., 2009, 2012; Nobre et al., 2015; Bejgarn et al., 2015; H.-X. Li et al., 2016b; Gandara e Silva et al., 2016) corroborate the hypothesis that additives are responsible for the toxicity we observed on oyster embryo mortality. For example, Nobre et al. (2015) revealed higher anomalous embryonic development in the sea urchin *Lytechinus variegatus* exposed to leachates from virgin pellets (66.5%) than from beached pellets (4.8%). An acute toxicity of leachates from 11 newly bought plastic products was also demonstrated on *D. magna*, causing mortality with a 48 h EC_{50} ranging from 2 to 235 g plastic. L^{-1} (Lithner et al., 2012). Although the marked difference in toxicity between new and aged plastic leachates was likely caused by additives from the new plastic gear, toxicity occurred in both types of plastic leachates and seems to be linked to specific compounds. Indeed, significant impairment of *P. margaritifera* embryo-larval development was observed in A-24 and A-120 at 100 g.L^{-1} compared with the control (no effect), while the latter had a concentration of phthalates five times higher (i.e. 2314 ng.L^{-1}) than in A-24 and A-120 (539 and 430 ng.L^{-1}, respectively). This suggests that (1) such a concentration of phthalates does not necessarily lead to toxic effects (at least here for the pearl oyster early life stages) and/or (2) that other compounds (either as additives or adsorbed chemicals) are responsible for the observed toxicity.

Toxicity from new plastic leachates is likely due to plastic additives, as these plastic gears had not been exposed to potential contaminants *in-situ,* in contrast to the aged ones. For the aged gears, the toxicity of their leachates seems to be linked to absorbed PAHs and pesticides from the environment. Toxic compounds, such as BaP, chrysene, σ- and γ-HCH, chlorpyrifos and σ-endosulfan (Fitzhugh et al., 1964; Paul et al., 2013; Menezes et al., 2017; Kopjar et al., 2018), were present in smaller quantities, down to completely absent, in the control and leachates obtained from new plastics compared to aged ones. For example, BaP is a priority pollutant (UE, 2008; US EPA, 2014) used as a reference PAH in ecotoxicology (Banni et al., 2017). BaP can be found throughout the marine environment and has been reported to cause oxidative stress, endocrine disruption and genotoxic effects in mussels (Gómez-Mendikute and Cajaraville, 2003; Banni et al., 2017). Furthermore, Sauter and Steele (1972) also demonstrated that γ-HCH (1.0 and 10 ppm in feed), a widely used organochlorine pesticide, significantly reduced hatchability, egg shell thickness and production, and increased embryonic mortality in the chicken. Thus, the presence of BaP and HCHs in aged plastic leachates may have, among

other contaminants, contributed to the alteration of the embryo-larval development in the pearl oyster. However, HCHs were also reported to have hormesis effect, as demonstrated with α-HCH (a biproduct in the manufacture of γ-HCH), when given at a low dose can protect against DNA damage and cytotoxic effects induced by subsequent exposures at a much higher dose (Fukushima et al., 2005). The theory of hormesis has been defined as a dose-response relationship in which there is biological activation at low doses but an inhibition at high doses, and *vice-versa* (Calabrese, 2002). Interestingly, the presence of γ-HCH in aged leachates (ranging from 2.2 to 29.7 ng.L^{-1}), especially in A-120 (20 times greater than N-24 and N-120), could have led to hormesis-type effect on embryo development as observed with complex variation between conditions, slightly higher in aged (91–97% from both A-24 and A-120) than in new (62–82% from both N-24 and N-120) plastic leachates (at 0.1, 1 and 10 g.L^{-1}), the control being intermediate (83%). This trend is similar to the *in-vitro* embryo development in mouse showing a slightly higher number of intact embryos and blastocystes after an exposition to γ-HCH at 3.6 μg ml^{-1} compared to control before a significant dose-dependent decrease at 7.2, 14.5 and 29 μg ml^{-1} (Alm et al., 1996). In our study, this hormesis hypothesis may explain the rapid development of trocophore larvae to D-stage occurred in A-120 at 100 g.L^{-1}.

Furthermore, the polymer nature of plastic gear also seems to play an important role in the toxicity we observed and has been demonstrated in previous studies. Lithner et al. (2012) observed that leachate from new consumer products made of PP showed no toxicity to *D. magna*, while leachate from PE gear appeared toxic (48 h EC50 at 17–24 g of plastic. L^{-1}). Similarly, H.-X. Li et al. (2016b) observed that the leachates from PP showed low toxicity to the barnacle *A. amphitrite* with a low effect on both larval survival (87–97%) and settlement (10–28%), but PE was more toxic (77–96% and 0–4%, respectively) to this species. In our study, synthetic rope made of PE and additives measured in leachates that seemed essentially to be coming from it could be the major source of the toxicity we observed. It would therefore be interesting to further assess the toxicity of leachates from synthetic rope (PE) and spat collector (PP), separately, so as to be able to dissociate the toxicities associated to each plastic type. Overall, toxicity assessment could be improved by individually testing identified chemicals at the measured concentrations on pearl oyster embryos in order to be able to dissociate singular implication of analytes in the toxicity we observed. Types of gear and polymer play therefore a major role in toxicity demphasizing the needs of testing plastics that animals mostly encounter in the wild as we did here.

4.3. Environmental implications in pearl-farming lagoons

Our work adds further information to the growing literature on the toxicity of plastics to aquatic organisms, especially through their associated leachates dissolved in seawater. We demonstrated experimentally that plastic pearl-farming gear can impair embryonic development in the pearl oyster, and it should be considered in an environmental context that synthetic ropes and spat collectors come into close contact with early life stage of *P. margaritifera*. As a consequence of plastic accumulation in pearl-farming lagoons, leaching chemicals, especially additives, could represent an increasing ecotoxicological risk for marine organisms (Hermabessiere et al., 2017). Although much higher desorption times are expected for plastic farming gear in farming atolls, this *in-vitro* study revealed high toxicity of leachates from both new and aged farming gears on embryo-larval development of *P. margaratifera*. As higher leaching times may likely release wider variety and higher quantity of toxic chemicals at least for aged plastics, there is a need to assess deeply effects and extend this question to productive Polynesian lagoons. Therefore, even if chemical analyses and concentrations of leachates tested may be difficult to interpret from an ecological point of view, the screening of plastics provides relevant toxicity information. Considering the very limited concentrations of phthalates in aged plastic leachates and the relatively short time of desorption tested here, our study shows their non-covalent association with the polymer matrix and therefore their potentially important desorption into seawater and natural environment. In the Venda region of South Africa, it was shown that plastic pollution caused river water pollution by phthalates at levels ranging from 0.16 to 10.17 mg.L-1 (Fatoki et al., 2010). Given the increase of both operational and derelict farming gear in pearl-farming lagoons, their accumulation could lead to a significant contamination by additives, including phthalates. Considering a 200-m long collecting station (rearing line) composed of spat collectors (39 kg) and synthetic ropes (135 kg), our estimated leaching of phthalates (i.e. 6.3 μg.g-1 and 184 μg.g-1, respectively) can hypothesized that a collecting station may release, at least, 25 g of phthalates during its use (about 3–5 years). Indeed, it would have been relevant to know the age of the aged plastics used here to refine this estimate, the curve of desorption over time, and the environmental factors knowing that aging is driven by many environmental factors. Extrapolating these data to the semi-enclosed lagoon of Takapoto (14°37′39″S, 145°12′18″W, FP) containing approximately 562 tons of rearing lines, may represent 80 kg of potentially leached and/or leachable phthalates (excluding leaching from other plastic gear such as buoys, nylon ties, grids and baskets), i.e. a phthalates contamination of 60 ng.L-1, considering the volume of the lagoon (i.e. 1.4 km3; Rougerie, 1979). Compared to our experiment, this overall assessment would amount to a mean leachate concentration ranging from 0.1 to 1 g.L-1. However, the toxic hazard associated with the leaching of phthalates should be considered contaminated hotspots. Indeed, despite progressive leaching, seawater dilution and potential photo- and biodegradation over time on the lagoon scale, future research should perform chemical analyses of *in-situ* seawater (using passive sensors to identify chronic contamination in a relevant and practical way) and test if accumulation areas of derelict plastic gear and rearing areas are becoming hotspots of phthalate desorption. Our results wholly suggest that the plastic gear used in the pearl-farming industry could release significant amounts of hazardous chemicals that may affect steps of pearl oyster reproduction, especially oyster spat settlement.

5. Conclusion

This *in-vitro* study shows that *P. margaritifera* embryo-larval development is affected by plastic leachates obtained from plastic pearl-farming gear that are closely interrelated with the early life stages of the pearl oyster. Operational and derelict areas of pearl-farming activity might be hotspots of chemicals raising the question of *in-situ* effects on cultivated oysters and other organisms. On a more global scale, the present study may alarm on the possible deleterious effects of aquaculture and fishing gears on marine life, highlighting the need to give greater consideration to the end-of-life of plastic materials used at sea. It represents a preliminary step to evaluate the impact of MPs from plastic pearl-farming gear on the pearl oyster, considering the biological changes caused by the MPs *per se*, chemicals incorporated into/onto the MPs, or both.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have

appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2020.115890.

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