BIOINDICATOR SPECIES OF PLASTIC TOXICITY IN TROPICAL ENVIRONMENTS

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Abstract

In French Polynesia, pearl farming represents the second economic resource of the country. The distinctive black pearls produced there are globally recognized and appreciated. However, pearl farms extensively use submerged plastic materials. Through gas chromatography coupled with tandem mass spectrometry detection (GC/MSMS) analysis, we were able to identify various POPs (Persistent Organic Pollutants) and additives released after 24 hours of leaching into seawater from these "pearl plastics" composed of PE (Polyethylene) and PP (Polypropylene). Subsequently, we tested different concentrations of this plastic leachate on five tropical species commonly raised in the pearl and aquaculture sector in Polynesia: *Pinctada margaritifera*, *Saccostrea cucullata*, *Holothuria whitmaei*, *Litopenaeus stylirostris*, and *Tripneustes gratilla*. Monitoring the embryo-larval development of these organisms allowed us

to establish a correlation between the decrease in the percentage of normal larvae and the plastic concentration. Through the use of regression models, the EC50 (Effective Concentration) of the plastic leachate for each species was determined, and demonstrated to range from 6.6 to 71.5g/L, depending on the species. The most sensitive species was the black teatfish *Holothuria whitmaei*, a tropical sea cucumber used for the first time for ecotoxicological tests. The sensitivity of this species, its large distribution in tropical areas, and the various advantages presented by its cultivation make it an interesting bio-indicator species for monitoring plastic pollution in tropical lagoons.

1. Introduction

The omnipresence of plastics in our current world is beyond dispute. Its versatility and low cost have made it an extremely advantageous material in various fields (transportation, construction, food, clothing, etc.). For about a century, plastic has been found everywhere, to the extent that the global annual production of plastic reached a record 390 million tonnes in 2021, representing a 4% increase from the previous year (Plastics Europe, 2022). Unfortunately, a portion of the produced plastic will end up in the oceans. In 2015, they estimated that between 4.8 to 12.7 million tonnes were being introduced into the oceans annually (Jambeck et al., 2015), this quantity was revised to be between 0.8 to 2.7 million tonnes (Meijer et al., 2021) and to 0.5 million tonnes with 49,000 to 53,000 tonnes consisting of microplastics (plastic particles <5mm) (Kaandorp et al., 2023).

Once in the sea or stranded, plastics are subjected to the combined mechanical action of water, photolysis related to ultraviolet exposure, and biological activity that promotes their abrasion, fragmentation, degradation, and biodeterioration, generating smaller pieces (Ali et al., 2021). Their abundance in the marine compartment is such that the presence of plastics is observed in sediment deposits in both shallow and deep waters (Zalasiewicz et al., 2016, review in Galgani et al., 2022). Thus, it is possible to geologically identify our contemporary era, the

Anthropocene, as the indelible imprint of human presence on Earth, through the presence of these elements in sedimentary layers. The Anthropocene is often referred to as the "Plastic Age." (Rangel-Buitrago et al., 2023). Their presence in the water induces pollution of a physical, biological, and chemical nature. Physical pollution results from the presence of macro or microplastics that can cause suffocation, strangulation, intestinal perforations, or disrupt the motility of plankton(Laist, 1997; Gall & Thompson, 2015; Garnier et al., 2022). Plastics can also lead to biological pollution by providing habitat and a means of transportation to invasive and/or pathogenic species through a phenomenon commonly referred to as the "raft effect" (Dussud et al., 2018; Haram et al., 2023). Finally, the third type of impact resulting from the presence of plastic in the marine environment is the release of chemicals from polymers. Indeed, upon immersion in the marine compartment, plastics will release various chemicals, including additives with kinetics depending on numerous parameters (Hahladakis et al., 2018; Luo et al., 2019; Paluselli et al., 2019). Some additives found in plastics, such as phthalates or pesticides like acetochlor, metolachlor, chlorpyrifos, are often implicated as endocrine disruptors. They may cause delays in growth, feminization of male individuals in certain species, as well as reproductive toxicity and carcinogenic effects (Mai et al., 2013;Guo & Wang, 2019;; Stara et al., 2019; Zhang et al., 2023).

At present, few studies focus on the impact of plastic leachates on marine species (Leistenschneider et al., 2023). Tests conducted typically use microplastics or nanoplastics to highlight the physical and mechanical risks induced by the presence of these microparticles in the environment. Therefore, the estimation of chemical pollution effects generated by the leaching of plastic materials is often neglected. Furthermore, most tested polymers are PS (Polystyrene), PE, or PVC (Polyvinyl chloride). The toxicity of PP materials is still underexplored in ecotoxicological tests, despite being the second most prevalent polymer on the ocean's surface (Leistenschneider et al., 2023) .

In 2022, French Polynesia exported 9 tonnes of pearls, generating 5 billion USD. This ranks the pearl sector in the second place on the economic resources (DRM, 2022). Pearl farms are largely using plastic materials, due to their low cost, resistance, lightweight, and effectiveness in larval capture. In tropical environments, plastics are exposed to higher temperatures, intense sunlight, and intense marine biological activity, having then shorter lifespans (Crusot et al., 2023). The extensive use of plastic, coupled with poor waste management practices, has made pearl farming one of the identified sources of macro and microplastic litter (Andréfouët et al., 2014). Estimates of the amount of waste in pearl lagoons by the local management bodies (DRM, 2015) revealed high concentrations of plastics in some pearl atolls, reaching up to 3,800 tonnes of marine litter in the single atoll of Takaroa (Tuamotus archipelago). Previous studies have also revealed the omnipresence of microplastics not only floating at the surface water, and in the water column of the lagoons, but also accumulated in the tissues of oysters in pearl atolls (Gardon et al., 2021). Recently, an estimate of the annual waste generated by pearl archipelagos, based on prevalent practices, reported more than 369 tonnes of waste per year for Mangareva island in the Gambier Archipelago (Crusot et al., 2023). This raises the question of toxicity of leachates from polymers used in pearl oyster aquaculture, and how they may affect the biodiversity of coral reef ecosystems. A preliminary study has demonstrated the lethality and possible teratogenic effects of plastic leachates from pearl materials on larvae from the black-lipped pearl oyster *Pinctada margaritifera* (Gardon et al., 2020). The percentage of abnormalities observed in these oyster larvae increases with the concentration of the tested plastic leachates. The same authors showed that leachates obtained from newly submerged pearl plastics for 24 hours had higher toxicity than leachates from aged plastics submerged for the same period. As a consequence, there is a need for toxicological tests adapted to the monitoring of plastic impact in tropical environments, further providing the scientific and technical basis for mitigation measures. Typically, good bioindicator species should possess several characteristics, such as a well-known biological cycle, homogeneous responsiveness to a pollutant, and the existence of identifiable toxic effects associated with the degree of pollution (Li et al., 2019). In recent years, numerous ecotoxicological tests have been conducted, generally using echinoderms or bivalves (His et al., 1999; Bonaventura et al., 2021), well adapted to temperate waters . Tropical species are largely underrepresented in the literature, or focusing on issues related to the toxicity of metals from mining (Gissi et al., 2018; Markich, 2021), particularly on coral species (Binet et al., 2023; 2018; Hédouin et al., 2016). For now, and while these species are of greatest interest for tropical aquaculture, no ecotoxicological tests had been conducted on tropical sea cucumber black teatfish *Holothuria whitmaei*.

Here, our study was aimed to (i) highlight the toxicity of PE and PP that are largely use in pearl aquaculture industry, (ii) to test various tropical species for the development of ecotoxicological tests adapted to the local context, and (iii) identify the best bioindicator species for chemical pollution induced by plastic leaching. Overall, this will support a better understanding of the impact of leachates from PE and PP plastic materials on the embryo-larval development of tropical species.

2. Materials et Methods

2.1. Plastics selection and leachate preparation

In this study, we specifically investigated the impact of chemical pollution induced by the immersion of new plastic materials used in pearl farms on marine organisms. We selected new ropes and collectors, which are among the most frequently used materials in pearl farming (Crusot et al., 2023). Macroplastics from new ropes and collectors were cut with scissors, sterilized with 70% ethanol, into smaller plastic particles of approximately 5mm. For leachate preparation, all glassware (flasks, filter funnels, Erlenmeyer flasks, borosilicate bottles) was autoclaved. The seawater used for all experiments was directly pumped in front of the Pacific

Ifremer Center (CIP) in the lagoon of Vairao (Tahiti, French Polynesia). It went through several filtration steps: strainer, sand filter, 25µm, 10µm, and 1µm pocket filters, followed by a double 1µm cartridge filter, UV sterilization, and finally autoclaving (sterile seawater - SW). Water parameters were measured using a multiparameter probe, and salinity was readjusted with MilliQ water if necessary (salinity: 36 psu; pH: 8.2). The leaching step was adapted from the protocol used by Gardon (Gardon et al., 2020). Four liters of pearl plastic leachate (PPL) and four liters of control leachate (CL) were prepared. For PPL, 200g of collector microplastics and 200g of rope microplastics were added to 4L of sterile seawater in a 10L flask. For CL, 4L of sterile seawater was placed in a 10L flask. Leaching was conducted at room temperature (26°C), under natural light, with a magnetic stirrer set at 600rpm. After 24 hours of leaching the materials in water, both solutions were filtered with GF/C filters (1.2 mm of porosity, Ø 90 mm, Whatman™). PPL and CL were aliquoted into borosilicate bottles wrapped in aluminium foil, each containing 250mL, and stored in the freezer at -20°C.

2.2. Polymer and chemical analysis

The identification of polymers in pearl plastic materials, specifically ropes and collectors used throughout our study, was performed using Raman spectroscopy (AlphaR 300 Raman micro-spectrometer, Oxford Instrument/WITec, LDCM/ Brest, IFREMER) equipped with two lasers (532 nm and 785 nm).

Chemical characterization of leachates was conducted in duplicate by CEDRE (Brest, France). using gas chromatography (HP 7890N, multifunction injection Combipal MPS2, Gerstel) coupled with tandem mass spectrometry (GC/MSMS). The interface temperature was set at 300°C, with a preprogrammed injection of 50°C (0.5 min) to 280°C (6 min) at 15°C/min, coupled with a temperature-programmed injector (Cooling Injection Device, Gerstel: -10°C (0.05 min) to 300°C (10 min) at 12°C/s). The temperature program of the oven was: from 70°C (0.5 min) to 150°C at 20°C/min, then 320°C (5 min) at 7°C/min. Helium was used as the carrier gas. The capillary column used was an RXi 5-ms (Restek, Bellefonte, USA). The chromatograph was coupled with a tandem mass spectrometry detector (Agilent 7000 Triple Quad). Quantitative analysis of PAHs, PCBs, PBDEs, pesticides, and additives was performed by internal standard calibration in MRM mode with two transitions for each compound (Quantifier/Qualifier). The acquisition frequency of each fragment was 2 cycles/s. For the analysis of organic compounds, chromatograms were reprocessed using MassHunter Workstation V10.0 software (Agilent Technologies).

2.3. Larval development and tests design

The method of induction through thermal shocks on oysters and sea urchins was adapted according to the protocol by Ky et al. (2015). The animals were kept at 24°C for 3 days and then rapidly immersed in a tank at 32°C (Ky et al., 2015). For osmotic shock performed on *Saccostrea cucullata*, oysters were air-dried and rinsed with fresh water (Coeroli et al, 1984). After thermal or osmotic shocks, individuals emitting gametes were isolated in individual beakers and sexed. Once the quality of the gametes was verified, fertilization was performed. Holothurians were obtained from the commercial hatchery Tahiti Marine Products (TMP), where gametes were naturally spawned. Males and females were mixed in holding tanks, and the water was then filtered, after fecundation, to concentrate the emitted eggs and adjust their concentrations. *Litopenaeus stylirostris* larvae were directly supplied to us at the nauplii 1-2 stage by the Centre Technique Aquacole of VAIA (Tahiti). The number of nauplii was adjusted through successive filtration, before immediate use. For all species, the fertilization percentage was estimated one hour after combining male and female gametes, through observations under a microscope and counting with a Malassez cell.

2.4. Microplate Tests

The contact between the larvae and the different solutions was carried out in untreated cell culture plates (TPP, Techno Plastic Products, Switzerland). The volume and concentration of larvae per well were optimized during the various pre-tests and adapted for each species (Table. 1). Once the larvae are added to the wells, the plates are kept in the dark in a Memmert climatic chamber to maintain a constant humidity level (64%rh) and a temperature chosen for each species.

2.5. Positive Control

Copper is often used as a reference toxicant because of its widespread use in various industries, its natural presence in the environment and its potential to cause adverse effects on aquatic organisms. Copper in the form of copper sulphate ($CuSO_4$) was diluted in sterile water and used as a positive control to monitor the quality of batches of larvae. The concentration ranges tested for each species will be expressed as equivalent Cu^{2+} concentrations in the remainder of this study (Table.A.2). The tests were performed in 5 replicates.

2.6. Negative controls

In our tests, we used two negative controls. Firstly, the control leachate (CL) allowed us to verify that there has been no contamination of our solutions during the preparation and conditioning phases of the leachates. Secondly, we also prepared a sterile seawater control to test the quality of seawater used as a diluent for PPL. The tests were performed in 5 replicates.

2.7. Ecotoxicological Tests, PPL

To test the potential toxicity of plastic leachates from pearl farms, we conducted a range of concentrations, expressed in grams of plastic per litre of seawater (g/L), ranging from 0.001 to 100 g/L (Table.A.3). Dilutions were made with sterile seawater and the toxicity test was conducted in five replicates. During the testing phases, the development of larvae was regularly monitored using a Leica DMI3000B inverted microscope. Considering the different developmental stages, we predefined a target stage for each species that was easily identifiable, and before the larvae started to feed to avoid interferences (Figure.1). When at least 80% of the larvae under control conditions had reached this stage, the test was stopped for all conditions by adding 10µL/mL of 8% formaldehyde per well. Larvae that reached the predefined target stage were counted in the "normal larvae" category. Any showing growth delay or malformation was counted as "abnormal larvae."

2.8. Determination of EC50

In order to compare the sensitivity of the five model species to plastic leachate in a consistent manner, we compared their effective concentration at which 50% of the larvae exhibit a developmental anomaly compared to the control condition (EC50). For each species studied, the EC50 values for PPL and $CuSO_4$ were estimated using predictive models. Dose-response curves were generated using the "drc: Analysis of Dose-Response Curve" package. Among the regression models available in "drc", the best-fitted model for each dataset was selected. Model selection was based on various criteria such as "Log-Likelihood," "IC" (Information Criterion), "Lack of fit", "Residual standard error," and "Variance of the residuals," which assess the quality of the fit for each model. The analyses were conducted using R 4.0.5.

3. Results

3.1. Nature of pearl farm plastics and leachates analysis

After Raman spectroscopic analyses, we identified the polymers constituting the ropes and collectors as polyethylene (PE) and polypropylene (PP) confirming the observations of Gardon et al., 2020. Chemical composition analyses of control leachate (CL) and pearl plastic leachate (PPL) revealed the presence of numerous compounds (Table. 2). The concentrations mentioned below result from the average of the concentrations from the two replicates. Firstly, in the CL, the only organic compound in a concentration high enough to be detectable was nonylphenols (NPs) with an average concentration of 13.53 ng/L (± 0.29).

For the PPL, four Polycyclic Aromatic Hydrocarbons (PAHs) were quantified: phenanthrene at 21.37 ng/L (± 0.54), pyrene at 6.93 ng/L (± 0.23), fluoranthene at 4.65 ng/L (± 0.06) and anthracene at 2.66 ng/L. The sum of the concentrations of PAHs is 35.6 ng/L (± 0.82). Numerous pesticides were also identified. In descending order of concentrations, they were: acetochlor 425 ng/L (± 25.67), metolachlor 78.37 ng/L (± 4.4), chlorpyrifos 27.03 ng/L (± 0.31), endosulfan alpha 3.92 ng/L (± 0.13), endosulfan beta 1.77 ng/L (± 0.07), Beta-BHC (β-Hexachlorocyclohexane) 1.35 ng/L (± 0.15), and gamma-BHC (gamma-hexachlorocyclohexane) 0.84 ng/L (± 0.02). Summing the concentrations of the 7 pesticides listed yields 538.7 ng/L (± 30.47). In the phthalate category, only Dibutyl Phthalate (DBP) was found, with a concentration of 169.46 ng/L (± 54.05). Finally, the last category of identified organic pollutants was made up of alkylphenols. Three were identified: NPs 88.36 ng/L (± 1.57), 4-tOP (4-tert-Octylphenol) 6.21 ng/L (± 0.69), and 4-OP (4-Octylphenol) 1.18 ng/L (± 0.01). The analyzed PPL thus contains 95.75 ng/L (± 2.25) of alkylphenols of three different types. No detectable level of PCBs or PBDEs was detected in any of the replicates.

3.2. Cu^{2+} as referent toxic

Five dose-response curves were obtained after exposure of *Pinctada margaritifera*, *Saccostrea cucullata*, *Holothuria whitmaei*, *Litopenaeus stylirostris or Tripneustes gratilla* larva to $CuSO_4$ pollutant (Fig. 3). Whatever the species considered, the rate of normally developed larvae in negative controls was above 80% which allowed to calculate EC50. The five species were ranked from the most to the less sensitive to cupper cation, as follows: *Saccostrea cucullata, Holothuria whitmaei*, *Tripneustes gratilla, Pinctada margaritifera*, *Litopenaeus stylirostris,* with EC50 values ranging from 16.4 to 119.1 µg/L. The linear regression model best suited to each data set, the EC50 obtained with this model and a range of EC50s obtained with estimates from the other models are specified in Table.3.

3.3. PPL Tests

The results of exposure tests on larvae of the five tropical model species to pearl plastic leachate are presented in Figure.3. The tested PPL resulted in malformations and growth delays for all considered species. However, the species appear to exhibit varying sensitivities to the PPL. Calculations of EC50 values allowed for ranking these species based on their sensitivity. *Holothuria whitmaei* emerged as the most sensitive species, followed by *Pinctada margaritifera*, *Tripneustes gratilla*, *Litopenaeus stylirostris*, and finally, the least sensitive to PPL was *Saccostrea cucullata*. The EC50 values for PPL ranged from 6.6 to 71.5g of plastic per liter of seawater. The summary of all obtained EC50 values for PPL is presented in Table.4 for each species, the linear regression model best suited to each data set, the EC50 obtained with this model and a range of EC50s obtained with estimates from the other models are specified in Table.4.

4. Discussion

In recent years, the number of studies on plastic pollution has exponentially increased. In order to compare the results highlighted by various research teams worldwide addressing this issue, it seems essential to establish an unique and harmonized protocol, such as ISO standards for other oyster species (ISO 17244, 2015). The identification of reference model species for each biotope is crucial for the overall comparison of the toxicity induced by plastic pollution as well as the use of referent toxic products as positive controls in order to be able to compare geographical distant ecotoxicology studies using the same model species. In this study, we have endeavoured to test several tropical species with the aim of defining criteria resulting from practical aspects (ease of obtaining breeders, ease of triggering spawning, larval capacity to develop in small volumes without water renewal, ease of identifying development stages with easily recognizable morphological characteristics) as well as toxicological relevance (sensitivity of species to tested toxins). The ultimate aim is to identify the best bioindicator species for chemical pollution caused by plastic leaching. The routine application of these simple tests could facilitate the establishment of a monitoring network to alert about the exceeding of pollution thresholds in high-risk areas such as pearl farming zones.

4.1. Contamination of the Control Leachate

To validate the leaching protocol and ensure there was no contamination during various preparation phases, control leachates were sent to CEDRE (France). Only the presence of Nonylphenol (NPs) was identified in the control leachates (13.2-13.9 ng/L). This contamination can have several origins. One possibility is that the measured NPs were present on the glassware used. Another possibility, the presence of NPs is attributed to the contamination of the Vairao lagoon, from which the seawater was pumped. NPs are widely used as surfactants, antioxidants, detergents, emulsifiers, among other applications and can be found in paints, pesticides, cleaning products, and are also used as plasticizers (Environmental Protection Agency, 2010a, Noorimotlagh et al., 2020). The concentrations measured in our control leachates remain far below the concentrations considered risky for the environment (1.7 µg/l) (Brooke and Thursby, 2005) and do not seem capable of affecting the larval development of the tested species (Hong et al., 2022). Other chemical compounds in the control leachate were below detection or quantification limits.

4.2. Variability of leachates products

Compared to a study performed in 2020 first highlighting the toxicity of leachates from pearl plastic materials (Gardon et al., 2020), and using the same protocol, in the same laboratory, leachate composition was found different, with the concentration of phthalates decreased from 6,680.92 ng/L in 2020 to 169.46 ng/L in our work, while pesticides were increased from 21.69 ng/L in 2020 to 538.71 ng/L in our experiment. Highlighting the challenges in reproducing plastic leaching tests and the importance of chemical characterization. The differences observed between the composition of these leachates may be due to a number of external factors. The main reason being the plastics themselves. Although the plastics used in these two studies were standard PE and PP ropes and collectors used in pearl farming, we cannot rule out a difference in the manufacturing processes during which additives have been intentionally added during the manufacturing of plastics to provide them with desirable physical or chemical properties, such as plasticizers, stabilizers, or flame retardants (Hermabessiere et al., 2017; Lithner et al., 2011), or they may have been absorbed during their transportation or storage (Wang et al., 2016). These factors could explain the differences in composition between these two plastic leachates produced with different batches of plastics several years apart.

4.3. Chemical Analysis of Pearl Plastic Leachates: Presence of POPs and Additives

Analytical techniques using gas chromatography coupled with tandem mass spectrometry (GC/MSMS) conducted on our PPL have revealed numerous persistent organic pollutants (POPs) and additives related to plastics. Among the identified POPs after 24 hours of leaching 100g of new pearl plastics in one litre of seawater, some have already been recognized as posing risks to marine ecosystems. The most represented class of POPs was pesticides and acetochlor was the main one. Among the noteworthy studies, Mahmood et al., (2021, 2022) demonstrated the numerous effects of acetochlor on the carp *Aristichthys nobilis*. Metolachlor, the second most prevalent pesticide in our leachates, has been linked to a significant increase in DNA and behavioural damage observed in oysters and crustaceans (Mai et al., 2012; Stara et al., 2019). Some research has highlighted the combined effect of pesticides and microplastics on copepods

(Bellas & Gil, 2020) or the synergistic effect of chlorpyrifos, acetochlor and dicofol on marine diatoms (Zhang et al., 2023).

The second largest group in our PPL was phthalates. Phthalates, like other plastic additives, are not bound to the polymer matrix of plastics and are therefore easily and rapidly leached into water during plastic immersion (Clara et al., 2010; Paluselli et al., 2019). After studying the effects of 18 different phthalates on microorganisms, algae, invertebrates, and fish, a toxicity ranking was established (Staple et al., 1997). Low molecular weight phthalates such as Dibutyl Phthalate (DBP) and Butyl Benzyl Phtalate (BBP) have higher acute and chronic toxicity than other tested molecules (Staple et al., 1997). A wide range of effects of DBP on marine organisms has been described, ranging from oxidative stress to enzymatic and metabolic alterations in marine animals at concentrations on the order of µg/L (Zhou et al., 2015). Teratogenic effects and increased mortality in *Danio rerio* (Ortiz-Zarragoitia et al., 2006) or feminizing the sexual characteristics of male fish and its impact on the gonadal development of juvenile fish (Bhatia et al., 2014) have occurred following exposure to DBP.

The predominantly found alkylphenol in our plastic leachates is nonylphenol. This chemical compound is identified as an endocrine disruptor, mimicking natural hormones such as 17β-œstradiol (P. C. Lee & Lee, 1996), which can impact the development, fertility and the production of female hormones in some fish (Kinnberg et al., 2000; H. J. Lee et al., 2003; Tabata et al., 2001).

Phenanthrene is the most toxic aromatic hydrocarbon tested by Black et al., its high toxicity is attributed to the large number of aromatic cycles it contains (Black et al., 1983). Since then, the toxicity of PAHs on the development of freshwater and marine fish larvae and juveniles has been demonstrated (Mu et al., 2014; Wessel et al., 2010).

When comparing the cited studies to the concentrations of additives measured in our leachates, certain distinctions become evident. Firstly, it is noteworthy that the orders of concentrations examined in previous studies were expressed in micrograms or milligrams per litre, whereas in our plastic pearl leachate (PPL), all concentrations were reported in nanograms per litre. But, with respect to xenoestrogens, a body of evidence from various studies has demonstrated that the effects of two or more compounds capable of disrupting the endocrine system may be additive or synergistic, even at low concentrations (Kwak et al., 2001; Rajapakse et al., 2002; Silva et al., 2002; Sol Dourdin et al., 2023). This observation is particularly significant given the intricate mixture present in our PPL, necessitating consideration of potential synergistic and/or cumulative effects.

4.4. Embryo-larval tests withcopper

The ISO standard suggests Cu^{2+} as a reference toxicant to ensure the standardization of ecotoxicological results. In our experiments, all examined species responded to the presence of this molecule, albeit in varying degrees. Previous studies indicate that the EC50 for copper ion in different bivalve species falls between 6.0 and 12.8µg/L (Markich, 2021; Nadella et al., 2009; Rosen et al., 2008). We found an EC50 for *Saccostrea cucullata* closely aligning with reference values, while the calculated EC50 for *Pinctada margaritifera* was found 3-fold higher than the EC50 range established by the same authors. Limited ecotoxicological studies, especially on tropical species, exist for sea cucumbers. Rakaj (Rakaj et al., 2021) and Morroni (Morroni et al., 2019) assessed the sensitivity of two species, *Holothuria tubulosa* and *Holothuria polii*, estimating their EC50 between 100 and 110µg/L for the first, and 260µg/L for the second. *Holothuria whitmaei* exhibited an EC50 of 30.2µg/L for Cu^{2+}, showing a greater sensitivity compared to other species within the same genus. The sensitivity of the shrimp to copper varies based on multiple factors including the life stage and exposure time. Tests on a freshwater species, *Macrobrachium rosenbergii*, estimated the LC50 to copper at 0.46 mg/L after 48h of exposure (Osunde et al., 2004). For *Litopenaeus stylirostris*, the copper EC50 was 119.1µg/L, and a higher concentration would be expected if "lethal concentration" had been chosen over "effective concentration". In *Paracentrotus lividus,* extensively studied in toxicology, exhibit similar EC50 estimates for copper ion, ranging from 60 to 70µg/L (Pétinay et al., 2009) and 45.8 to 72.8µg/L, depending on exposure time (Morroni et al., 2018). Other sea urchin species, such as *Echinometra mathaei* (29.85µg/L ± 1.86) and *Strongylocentrotus purpuratus* (14.3-20.6µg/L), display varying sensitivities. In our study, the tropical species *Tripneustes gratilla* had an EC50 of 33.1µg/L, consistent with estimates from published studies.

Comparing the EC50 for Cu^{2+} among model species in this study with those from prior research reveals valuable insights. Methodology variations significantly influence sensitivity measurements, and even species within the same phylogenetic "order" can exhibit different sensitivities. This is evident in *Pinctada margaritifera* and *Saccostrea cucullata*, both belonging to the *Ostreida* order, with respective EC50 values of 35.6 and 16.4µg/L for Cu^{2+}. Sensitivity appears highly species-dependent, emphasizing the need for future ecotoxicological projects to focus on tropical species, which may demonstrate varied sensitivities compared to their temperate counterparts.

4.5. Embryo-larval tests with PPL

The overall results indicate a significant effect of leachate from pearl plastic on all studied species. The increase in plastic concentration is associated with a rise in growth anomalies, such as malformations and developmental delays. The EC50 values obtained from projections of different models adapted to each species show varying effective concentrations of leachate from pearl plastics. The most sensitive species to PPL is *H. whitmaei*, followed by *P. margaritifera*, with an *T. gratilla*, *L. stylirostris* and *S. cucullata* as the least sensitive species.

The effective concentrations of plastics obtained for all species, on the order of grams per liter, appear to be distant from concentrations previously measured in the natural environment. However, plastic concentration at both global and local scales is heterogeneous and subject to numerous external constraints. Among the notable factors affecting plastic accumulation, size, polymer density (Guo & Wang, 2019b), extreme climatic conditions such as storms, hurricanes, flooding (Thompson et al., 2005), salinity (Lima et al., 2014), and hydrodynamics (Law et al., 2010) will concentrate plastics in preferential zones (reviewed by Thushari & Senevirathna, 2020). In the Pacific Ocean, Eriksen et al.,(2013) conducted samplings of the surface layer of the South Pacific subtropical gyre using manta nets. The average abundance measured was 70.96 grams of plastics per square kilometer. This high concentration is due to the zone associated with the convergence of surface currents, driven by local winds. More locally in Polynesia, campaigns estimating pearl farm waste have highlighted lagoon areas with high concentrations of waste corresponding to either old or still active pearl farming concessions or unauthorized areas. The work of Crusot et al., (2023) confirms this observation and documents 779 collection lines for 141 hectares of marine concessions for the single atoll of Takapoto, one of the most important pearl farming atolls currently. This high concentration of pearl material generates nearly 38.9 ± 3.4 tons/year of plastic waste annually. "Wild" waste identified in this atoll was mainly recorded in the South and West of the lagoon (DRM, 2015). Thus, local plastic concentrations can be much higher than the global estimates generally made at the lagoon scale (Gardon et al., 2021). In the event of suspected contamination of the lagoon, and in addition to ecotoxicological tests on larvae, it would be relevant to chemically characterise the water using passive sensors (Bartelt-Hunt et al., 2011; Net et al., 2015; Van Metre et al., 2017). In this case, the chemical compounds accumulated in the membranes could provide information about the origin of the contamination.

With regard to the technical and logistical aspects of reproduction mentioned in this study, we can state that species such as *P. margaritifera* and *T. gratilla* represent a challenge with regard to the conditioning of broodstock, with the aim of inducing spawning, as well as the success of fertilisation and the hatching rate of eggs. Ongoing research programs at CIP focus on optimizing breeding and conditioning for inducing spawning in these species. *L. stylirostris* presents a different set of challenges. In shrimp farming, spawning induction requires a complex and technical procedure. Although fertilization rates were good in various tests with this species, the hatching rate was low. To avoid compromising water quality in the reduced volumes of microplates, we chose to collect viable larvae after hatching instead of fertilized eggs, some of which might be non-viable and lead to an increase organic matter and potential contamination. However, this choice limited the contact time between the larvae and the PPL, beginning only 8 hours post-hatching.

Based on our experience, we can conclude that the tested species that were the easiest to raise and reproduce were *S. cucullata* and *H. whitmaei*. Due to its developed adaptive capabilities, *S. cucullata* has spread worldwide and thrives in both temperate and tropical environments (Do Amaral et al., 2020; Pagenkopp Lohan et al., 2015; Ramadhaniaty et al., 2018; Ulman et al., 2017). Unlike representatives of this species in temperate environments, specimens of *S.cucullata* in tropical environments are capable of reproducing throughout the year (Legat et al., 2021). This characteristic allows for the production of *S. cucullata* larvae at any season, which is a major advantage for implementing routine environmental monitoring. The minimum size for first maturation of this species has been determined to be 32.8 mm and 28.3 mm for females and males, respectively (Mafambissa et al., 2023). To select mature breeders, especially if collecting wild individuals is considered, it is advisable to choose individuals whose shell length exceeds these sizes. In our spawning attempts, a brief emersion phase of the breeders followed by a rinse with fresh water before immersion in seawater has always been sufficient to trigger spawning in several breeders with fertilization and hatching rates exceeding 90%. However, other reproduction inductions exist and have proven effective for the *Saccostrea* genus, such as the combination of decreased salinity with the addition of sperm to the breeders' water (Nowland et al., 2021).

In these tests, we also measured the potential of sea cucumbers *H. whitmaei* and the numerous logistical advantages that this species represents. This species, widely distributed in the Indo-Pacific, is highly present in French Polynesia. Research currently conducted by the company TMP contributes to increasing general knowledge about this species to enhance its breeding for pharmacological purposes. At present, limited information is available for this species. However, data on the breeding methods of sandfish sea cucumbers *H. scabra*, a tropical sea cucumber species with high market value belonging to the same genus, are available. The ideal weight for a mature breeder is 500g (Agudo, 2006). According to the same author, several spawning induction protocols have already been tested. Among the most effective and non-lethal combinations, it is recommended to air-dry the animals, followed by a cold and then a hot thermal shock. Alternatively, natural spawns can be obtained by collecting the animals just before the full and new moons, as was our case, with the peak fertility of this species identified during the austral summer. Eggs obtained from natural spawns are generally more numerous, but the quantity of oocytes emitted by induction, even outside the natural spawning periods, is on the order of 1 to 2 million per female (Agudo, 2006), which is more than sufficient for conducting tests in microplates

In the perspective of larval production for the implementation of ecotoxicological tests in tropical regions, we recommend using *S. cucullata* and/or *H. whitmaei*. To limit infrastructure, maintenance, and food production, we advise collecting a few breeders from the wild and employing non-lethal spawning induction methods so that these adult individuals can be returned to the natural environment once spawning is completed.

If we now compare the response of these two selected species in terms of their sensitivity to plastic leachate, it is observed that the 50% development anomalies are reached at concentrations of 6.6 and 71.5g/L of plastic for *H. whitmaei* and *S. cucullata* respectively. *H. whitmaei* is therefore almost 11 times more sensitive than *S. cucullata*. Considering these biological characteristics and the sensitivity demonstrated by *H. whitmaei* during exposure tests to plastic leachate, this species appears to be a good bioindicator of plastic pollution in tropical environments. The control of larval development in *H. whitmaei* opens the possibility of establishing a monitoring network for assessing pollution levels in pearl farming sites or monitoring the toxicity of aquaculture effluents in Polynesia. More broadly, the significance of this approach lies in the applicability of the results to the specific context of tropical island environments, particularly in lagoons and aquaculture areas.

For the future, it would be interesting to continue the development of microplate tests by confronting the larvae with multistress and thus better assessing their behaviour in the face of current global changes. Indeed, synergistic effects have been demonstrated between the effects of climate change and marine pollution (reviewied by Cabral et al., 2019). Synergistic effects are defined as stressors interplay resulting in a combined effect greater than the sum of their individual effects (Folt et al., 1999). The effects of increasing temperature or ocean acidification (Cao et al., 2018), two effects of global climate change, combined with metals or POPs (Su et al., 2017) or with plastique pollution (Bertucci & Bellas, 2021) have shown more significant deleterious effects on marine organisms than the effect of each stress evaluated separately.

5. Conclusion

In to this study, we identified the composition of leachate from ropes and spat collector oysters made of polyethylene and polypropylene plastic. The results suggest that pearl plastics leachates are complex mixtures of POPs and additives such as hydrocarbons, pesticides, phthalates and alkylphenols. These chemical compounds prove toxic even at low concentrations (ng/L). Indeed, thanks to acute exposure toxicity tests developed in microplates, the harmful effects of plastic pollution on the early stages of development of tropical organisms have been demonstrated. Among the studied species, *Holothuria whitmaei* had never been used for ecotoxicological testing. In addition to its effective sensitivity to plastic pollution, this species has logistical and biological advantages that make it an interesting bio-indicator species to develop for monitoring chemical contamination of tropical lagoons. The range of sensitivity to the reference toxicant Cu^{2+} has been established for *H. whitmaei* and could serve as a reference for future ecotoxicological tests. As the use of plastics in aquaculture and pearl farming is chronic and massive, further studies are now needed to provide information on the impact on exposed organisms over generations and to characterise possible synergies between mixtures of POPs and additives and environmental factors set to change with current climate change.

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