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Plastic pellets make *Excirolana armata* more aggressive: Intraspecific interactions and isopod mortality differences between populations

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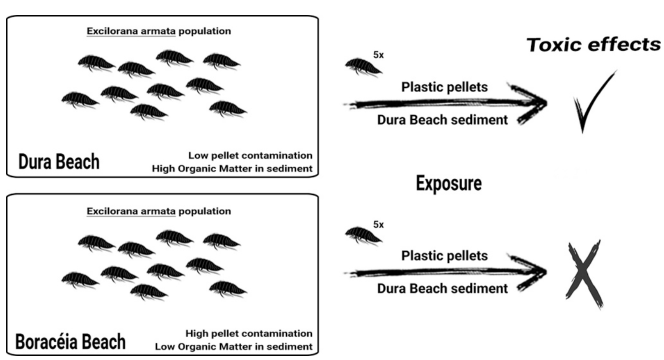
HIGHLIGHTS

- Plastic pellets increase necrophagy and cannibalism in *Exicirolana armata.*
- Tolerance to contaminants was different for distinct populations of *E. armata*.
- *E. armata* was more sensitive in beaches free from pellet contamination.
- Sediment quality should be considered during pellet toxicity assessment.
- Yellowish-beached pellets tend to be more toxic than white pellets.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic pellets represent a significant component of microplastic (< 5 mm) pollution. Impacts caused by plastic pellets involve physical harm and toxicity related to ingestion and non-ingestion (such as the release of chemicals

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in leachates). The latter is the main route of exposure for invertebrate macrobenthic populations. This study aimed to compare the toxicity of plastic pellets in distinct marine macrobenthic populations, considering the influence of sediment characteristics (organic matter and grain size) and quality (contamination by hydrophobic chemicals) on ecotoxicological effects, as well as the influence of color on the toxicity of beach-stranded plastic pellets. We performed three experiments on plastic pellet exposure using *Excirolana armata* from beaches with high and low pellet density. When exposed to pellets, populations that inhabit beaches without pellets demonstrate higher mortality than those inhabiting beaches with high pellet densities. The mortality of *E. armata* to pellets was higher when the exposure occurred in sediment with high organic matter (OM), suggesting that chemicals were transferred from pellets to OM. Yellowish beach-stranded pellets induced higher mortality of *E. armata* than the white tones did. We also observed lethargic (near-dead) and dead individuals being preyed upon by healthy individuals, a cannibalistic behavior that raises an ecological concern regarding the negative effects of this exposure on intraspecific interactions in marine macrobenthic populations.

1. Introduction

Microplastics (plastic pieces with a diameter smaller than 5 mm) are potentially toxic to marine organisms, although their effects on the environment are still not well understood. Plastic pellets (or nurdles) consist of primary microplastics, which are used as raw materials for manufacturing plastic objects. These particles are commonly and constantly released into the environment because of unintentional losses. About 90 tons of plastic pellets (the equivalent of ~2.7 million 1.5 L nonreturnable PET bottles) are estimated to be lost annually by the plastic industry (Lechner and Ramler, 2015), unintentionally spilled into the environment during manufacturing and transportation (Ogata et al., 2009; Corcoran et al., 2020). These spills often fall into runoff systems and rivers, which carry pellets toward the coastal environment (Ogata et al., 2009; Karlsson et al., 2018), where they tend to accumulate mainly on sandy beaches (Moreira et al., 2016; Corcoran et al., 2020).

Newly manufactured plastic pellets are usually white or translucent and can be called virgin pellets (Hammer et al., 2012). These pellets frequently contain high concentrations of chemical substances that are incorporated into polymers during industrial processes (Hammer et al., 2012; Andrady and Rajapakse, 2016; Yamashita et al., 2021). Once plastic pellets are lost, these chemical additives can quickly leach into the environment (Teuten et al., 2009; Nobre et al., 2015). Unlike virgin pellets, beach-stranded plastic pellets are usually yellowish or brownish owing to degradation (Karapanagioti and Klontza, 2007). Long periods of exposure to the environment darken the yellowish tone of the plastic pellets to shades of orange and brown. This color gradient is derived from hydrophobic chemicals present in the pellets or their degradation products, which are generally high present in dark tones (Endo et al., 2005; Fotopoulou and Karapanagioti, 2012; Yamashita et al., 2018). These plastic pellets hydrophobic chemicals can reach concentrations higher than those found in ocean waters (Andrady, 2011; Mato et al., 2001). Thus, the pellet color grade can be used as an indicator of chemical contamination (Endo et al., 2005; Izar et al., 2022a).

Marine invertebrates from infauna constantly interact with microplastics in contaminated coastal environments. Benthic organisms can bury microplastics into deeper layers of sediment during bioturbation and burrowing (Näkki et al., 2017; Gebhardt and Forster, 2018; Capparelli et al., 2022). Laboratory assays have shown acute and/or chronic toxicity of plastic pellet leachates to mussels (Gandara e Silva et al., 2016), sea urchins (Nobre et al., 2015; Izar et al., 2019), and sand dollar embryos (Albanit et al., 2022), copepods and amphipods adult organisms (Izar et al., 2019). The sublethal effects of microplastic exposure have been observed using biomarkers in oysters (Nobre et al., 2020) and crabs (Silva et al., 2022; Nobre et al., 2022). A first attempt at *in-situ* ecotoxicological tests for macrobenthic invertebrate populations was performed on a pristine sandy beach using real-world plastic pellet densities exposed to *Excirolana armata* (Isopoda) individuals, which caused toxic effects at all tested densities (Izar et al., 2022b). Isopods are ecologically important because of its ability to transfer plastic particles to higher trophic levels in food chains (Anbumani and Kakkar, 2018). As a shortcoming of our previous work (Izar et al., 2022b), *in-situ* experiments can introduce numerous environmental confounding factors, mainly with regard to the bioavailability of hydrophobic contaminants in the sediment. The properties of the local sediment can influence or mask toxic effects on organisms, placing the sediment as a factor and a natural stressor that can act in a multiple stressor toxic chain (Maulvault et al., 2019; Wieringa et al., 2022). The bioavailability of chemical compounds associated with the leachates of plastic pellets may vary according to the matrix and its properties to which the organisms are exposed, *i.e.*: sediment organic matter content (OM), among others (Koelmans et al., 2016).

The cirolanid isopod *E. armata* is abundant and ecologically dominant on sandy beaches with fine granulometry throughout South America, ranging from Rio de Janeiro to Northern Patagonia (Defeo et al., 1997; Lercari and Defeo, 2003; Lozoya et al., 2010). The preference for fine sand is an important factor in determining the exposure of this species to microplastics, as such particles (including plastic pellets) tend to accumulate in fine grain-sized areas (Enders et al., 2019; Corcoran et al., 2020; Vermeiren et al., 2021). Furthermore, it has been reported that micrometer-sized microplastics are ingested by *E. armata* (Vermeiren et al., 2021). However, this species is highly resistant to environmental stress and human activities (Lozoya and Defeo, 2006; Thompson and Sánchez de Bock, 2007; Lozoya et al., 2010; Gandara-Martins et al., 2015; Fanini et al., 2017; Laurino and Turra, 2021) and fits perfectly as a model organism. This has been highlighted in previous studies testing the responses of species to environmental stress (Laurino et al., 2020; Laurino and Turra, 2021) and microplastic pollution (Izar et al., 2022b). Once *E. armata* is affected, the entire trophic chain may be compromised because of its importance as a primary consumer (Lercari et al., 2010; Bergamino et al., 2011; Costa and Zalmon, 2017). This high resistance of the species leads us to a possible adaptation facility to stressors or natural selection. In mosquitoes, selection resistance has already been observed for insecticides, with some adaptive mechanisms, such as cuticle modification, increased detoxification enzymes, and target-site mutations (Nkya et al., 2013). As isopods are arthropods, they may experience acquired resistance. These observations led us to believe that populations on beaches that are more contaminated by plastic pellets present different responses to toxicity. Acute toxicity (mortality), as described above, is one of these responses and another one are some adaptive biological responses, or sublethal effects. These sublethal effects are early biochemical warnings of toxic effects that it has been studied by biomarkers neurotoxicity and oxidative stress to microplastics (Barboza et al., 2020; Nobre et al., 2020).

In the present study, we aimed to understand why different populations of the macrobenthic cirolanid isopod *E. armata* respond differently to plastic pellet stress depending on the environment in which they inhabit. To do this, (1) we exposed two *E. armata* populations from different sites to plastic pellets under equal conditions to verify differences in toxicity between populations. Here, we tested the hypothesis of acquired resistance (natural selection) of this species to plastic pellets when inhabiting beaches with high densities of these particles. The sublethal effects of this exposure in both populations were measured by analyzing biomarkers for neurotoxicity (cholinesterase-like activity) and oxidative stress (lipid peroxidation and DNA damage). (2) We also exposed a single population of *E. armata* to different beach sediments, in order to test the influence of sediment characteristics (OM and grain size) and quality (organic and metal contamination) in the ecotoxicological bioassays. Here, we expected that higher OM, fine granulometry and/or contamination would induce more toxicity when associated with plastic pellets, a multiple-stressor phenomenon, owing to the high affinity of hydrophobic contaminants to organic matter, making them more bioavailable. Finally, (3) we tested the toxic effects of the different beach-stranded plastic pellet colors under the hypothesis that darker pellets are more toxic than white tones. Results are critically discussed.

2. Methods

In this study, we performed three experiments exposing individuals of two distinct populations of *E. armata* to plastic pellets. The first population inhabits the Dura Beach in Ubatuba city (23°29′41″ S, 45°10′21″ O - Fig. 1), on the Northern coast of São Paulo state (Southeast Brazil). Dura Beach is a microtidal (tidal range < 2 m) dissipative beach with about 1700 m in length, sheltered at the deeper portion of a large bay, with a higher concentration of OM due to the presence of an estuary on one of its sides (Laurino et al., 2020; Izar et al., 2022b). It is located 200 km away from Santos Port, the largest port in Latin America, and the main source of plastic pellets for the entire coast of São Paulo (Izar et al., 2019). Once the beach is far from pellet sources, it is considered to be free from plastic pellet contamination (Moreira et al., 2016; Izar et al., 2019). In the last decade, Dura Beach was rated as regular/good for bathing (CETESB, 2020a) and is considered a moderately impacted area, likely due to the presence of marinas and boat traffic in the region (CETESB, 2020b). Even with its proximity to state park (*Parque Estadual da Serra do Mar*), this protected area is restricted to mountainous areas on the coast, allowing construction and real estate speculation on the sandbanks of the region's beaches. The sediment of the region is moderately contaminated by polycyclic aromatic hydrocarbons (PAHs) from biomass combustion sources (Moreira et al., 2021) and contains a considerable amount of sewage, especially in the summer during the high tourism season (CETESB, 2019).

The second population inhabits the Boracéia Beach, in Bertioga city (23°45′25″ S, 45°49′8″ O - Fig. 1), on the middle coast of São Paulo state. It is close to the Santos port (~ 50 km) and has a moderate pellets density (Izar et al., 2019; Izar et al., 2022a). Boracéia Beach is also a microtidal (tidal range < 2 m) dissipative beach with a gentle slope and fine granulometry (Laurino and Turra, 2021). However, it is longer than Dura Beach, with about 8000 m in length. Its sediment is relatively uncontaminated by inorganic and organic elements/compounds, owing to the low input of sewage (CETESB, 2019), the proximity of a State Park (*Parque Estadual Restinga de Bertioga*) and a Marine Protected Area (Área de Proteção Ambiental Marinha Litoral Centro - APAMLC) and for the characteristics of an exposed beach. Boracéia Beach was rated as good for bathing in most of the years of the last decade (CETESB, 2020a). Furthermore, Boracéia Beach is located in the same bay as Itaguaré Beach, whose sediment is considered a control site for contamination in ecotoxicological studies (Ferraz, 2013).

The beach-stranded plastic pellets used in all experiments were

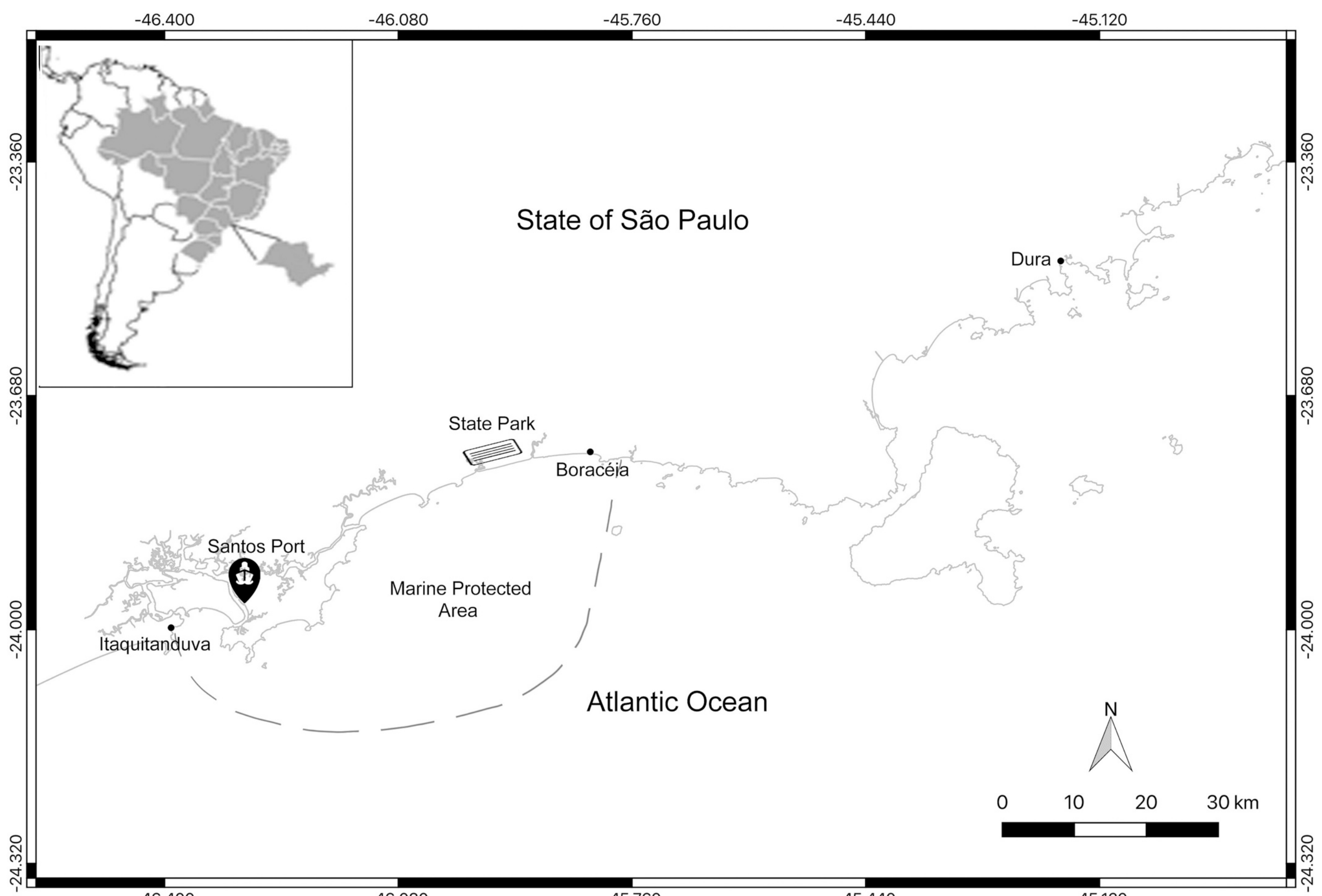

Fig. 1. Map of the São Paulo north and center coast (Brazil) showing the locations of the three beaches used in this study and their distance to the Santos port. Plastic pellets were collected from Itaquitanduva Beach. The beach sediment and individuals of *Excirolana armata* used in the experiments were sampled at the Boracéia and Dura beaches. Dashed line represents the Marine Protected Area and the rectangle the State Park.

sampled at Itaquitanduva Beach in September 2021. This beach is located on the west side of Santos Bay (23°59′50″ S, 46°23′28″ O - Fig. 1), close to the Santos Port and to the major industrial complex of Cubatão (8 km far from the mouth of the Santos Channel, where Santos Port is located, in the east side of the Santos Bay). This beach is affected by the discharge of plastic pellets and chemical contaminants (Moreira et al., 2016; Taniguchi et al., 2016; Izar et al., 2019; Izar et al., 2022a). Itaquitanduva Beach has a high pellet density that is comparable to the highest in the world (Izar et al., 2022c). The plastic pellets from Itaquitanduva Beach presented high concentrations of adsorbed hydrophobic contaminants, such as dichlorodiphenyl trichloroethanes (DDTs), polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), and polychlorinated biphenyls (PCBs) (Taniguchi et al., 2016; Ohgaki et al., 2021), which were found in extremely high concentrations (> 500 ng g^{-1} of pellets) (Ohgaki et al., 2021).

Plastic pellets were picked from the sandy beach surface sediment along the high tide line by active searching, visually identified, and collected. The beach-stranded pellets sampled (~ 8 thousand) were stored in glass containers, regardless of color or polymer type, and kept refrigerated (5 to 10 °C) until the exposure in experiments. To simulate the distribution of plastic pellets in the natural environment, collected pellets were randomly selected for each exposure.

2.1. Different populations under the same conditions

To ensure the same conditions for both *E. armata* populations tested in the bioassays, in September 2021, we exposed them to the same sediment from Dura Beach. The sediment of this beach was chosen because it has a higher level of PAHs contamination and sewage (CETESB, 2019 and 2020a), allowing us to test the effect of multiple stressors (plastic pellets and contaminated sediment).

At Boracéia Beach, we collected about a thousand individuals of the macrobenthic isopod, finding the tracks left on the sand surface, digging those tracks, and sifting the sand to collect the organisms. We visually selected only adult individuals (> 3 mm in length; Petracco et al., 2010) and stored them in a plastic bucket with sifted local sediments and seawater for transportation. In the laboratory, the organisms collected at Boracéia Beach had one day of acclimatization with constant oxygenation due to the transport stress. The population of Dura Beach was sampled following the same methods described for the population of Boracéia Beach. After sieving, individuals had 1 h of acclimatization due the stress of collection management and placed in beakers to be exposed to the assay.

Dura Beach sediment was sieved to remove organisms from the samples used in the bioassays. A total of 20 mL of sieved sediment was added to a 50 mL glass beaker and local seawater was added until a thin layer of water covered the sediment. We exposed both populations of *E. armata* to two treatments: (1) control – no pellets, and (2) pellets – a very high pellet density (40 pellets) was added to the sediment surface layer per beaker, which was equivalent to twice that found in Hawaii by Mcdermid and Mcmullen (2004). This pellet density was chosen to ensure that the toxic effect would occur and to simulate a future catastrophic plastic pellet density.

In each treatment, five individuals of the same *E. armata* population were added to each beaker and exposed for 6 h. At the end of the exposure period, the sediment from each beaker was sifted, and the surviving individuals accounted for in each treatment and population. Missing individuals were considered dead because the cannibalistic behavior of the species had previously been observed in similar experiments (Izar et al., 2022b), and it is highly unlikely that the tested organisms would escape the beakers. The experiment had ten replicates for each group and was repeated thrice, totaling 120 replicates ($n = 30$ per treatment).

A Generalized Mixed Model (GLMM) with a Negative Binomial distribution was built for mortality, considering the number of dead or missing individuals (the dependent variable). Three factors were tested in the model: Date (Factor 1, random, 3 levels), Beach population (Factor 2, fixed, 2 levels), and Treatment (Factor 3, fixed, 2 levels). For all statistical analyses, we adopted a confidence level of $p \leq 0.05$, and the distribution used in the models was determined by the best fit to the model (a smaller Akaike information criterion - AIC, and the normality of residuals). The rate ratio (RR) indicates the effect size with a 95 % Confidence Interval (CI95%). All statistical analyses were performed using the open-source statistical software Jamovi (2021).

The sublethal effects of exposure were determined by biochemical biomarker analyses. At the end of the experiment, surviving *E. armata* were immediately frozen on ice, transported for 4 h to the laboratory, and subsequently stored in an ultrafreezer (−80 °C). We analyzed the neurotoxicity biomarker cholinesterase-like (ChE) and two biomarkers of oxidative stress, lipid peroxidation (LPO), and DNA strand break (single break) - DNA damage.

Neurotoxic effects were evaluated based on ChE-like activity, using the method proposed by Ellman et al. (1961) and adapted by Herbert et al. (1995). We added a solution of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and acetylcholine iodide to each sample, and the absorbance was measured at a wavelength of 415 nm. The results were expressed as nmol min^{-1} mg^{-1} of total protein.

LPO was evaluated based on the protocol described by Wills (1987), in which thiobarbituric acid reactive substances (TBARS) and tetramethoxypropane standards were added and diluted in a homogenizing solution. The measurement was performed using fluorescence, in which an excitation wavelength of 516 nm and an emission wavelength of 600 nm were applied. The results were expressed in μM TBARs mg^{-1} of total protein.

DNA damage was evaluated using the alkaline precipitation method proposed by Olive (1988). We applied a salmon sperm DNA standard curve for damage standardization and used Hoechst 33342 solution to provide luminescence to the sample. Damage was quantified by fluorescence, using a wavelength of 360 nm for excitation and 450 nm for emission. The results were expressed as μg DNA mg^{-1} of total protein.

The total protein content used to normalize the biomarkers data was evaluated using the Bradford method (1976). Each replicate of the biomarker analyses (10 organisms) was composed of 5 organisms from each of the two replicates of the previous experiment. Thus, biomarker analyses had five replicates for each group (beach and treatment) and were performed for the last two days of the experiment (days 2 and 3), totaling 40 replicates ($n = 10$ per treatment).

For ChE and LPO, a Generalized Mixed Model (GLMM) with Gamma distribution (link function = Identity) was built with three factors: Date (Factor 1, random, 2 levels), Beach population (Factor 2, fixed, 2 levels), and Treatment (Factor 3, fixed, 2 levels). For DNA damage, a General Mixed Model (GMM - Linear distribution) was built using the same statistical software with the same previous three factors.

2.2. Different sediments for the same population

To test the influence of sediment characteristics and quality as a factor for toxicity, we repeated the previous assay, following the same methods and experimental design, with a standard population exposed to the same pellet density. This test was performed using the Boracéia Beach population of *E. armata* in sediments from Boracéia and Dura beaches. The population of Boracéia Beach was chosen because it is resistant to the stress of plastic pellets in their natural environment (sediment), according to the results of our first experiment. The experiment had ten replicates for each group, and was repeated three times per beach sediment, totaling 120 replicates ($n = 30$ per treatment).

A Generalized Mixed Model (GLMM) with a Negative Binomial distribution was built for the dependent variable mortality with three factors tested: Date (Factor 1, random, 6 levels), Sediment (Factor 2, fixed, 2 levels), and Treatment (Factor 3, fixed, 2 levels).

As the organisms were tested in different sediments, it was considered that sediment properties could be a confounding factor. Therefore, to address the possible influence of OM during pellets exposure, sediment was collected from both beaches in 10 random replicates. In the laboratory, OM was measured in the dried sediment (60 °C for 48 h) by weight loss after incineration (550 °C for 6 h) for each sample. To determine the grain size (Φ), samples were sifted following the procedure proposed by McCave and Syvitski (1991), and classified as parameters following Folk and Ward (1957). We sifted the dry sediment into six different granulometric fractions (2, 1, 0.5, 0.212, 0.125, and 0.063 mm) for 15 min at a speed of 3600 bpm and individually weighed each fraction. Sediment properties from both sites were statistically compared using the Student's *t*-test and Welch's correction, if necessary.

Beach sediments were also analyzed for organic compounds and mercury (Hg), to address the possibility that sediment contamination influences isopod mortality when associated with pellet exposure. Three sediment samples from each beach (Dura and Boracéia) were collected, stored in calcined aluminum containers, refrigerated in ice, and transported to the laboratory, where they were kept frozen (−14 °C to −25 °C). The sediments were lyophilized in the laboratory. For chemical analyses, we prepared samples using a miniaturized solid–liquid extraction method (Santos et al., 2016, 2018), in which 25 mg of sediment from each beach sample was added to a microextraction device (Whatmann Mini ™ UniPrep Filters without syringe, Whatmann, USA) with 500 μL of a mixture of solvents (18 % acetonitrile +82 % dichloromethane) and sonicated for 23 min. The final filtered extract was injected into a gas chromatograph coupled to a mass spectrometer (GC–MS QP-2010 ULTRA, Shimadzu, Japan) to analyze PAHs and their derivatives (oxy- and nitro-PAHs). The condition of the GC–MS is described in Santos et al. (2016). Quantification was performed using the calibration curve for PAHs. The limit of detection (LOD) and limit of quantification (LOQ) of each analyte were calculated following the International Union of Pure and Applied Chemistry (IUPAC) (Thompson et al., 2002): LOD = (3SE/α) and LOQ = (10SE/α), where SE is the standard error of the linear regression and α is the slope of the linear regression of the calibration curve (all these information are in the supplementary data, Table S1). LOD was applied for each analyte. Analytical quality control parameters (evaluating, precision and accuracy) followed the method developed for sediments in Santos et al. (2018), in which reference certificated marine sediment deuterated standards (fluorene-D10 and pyrene-D10) were added to determinate control parameters and repeated ten consecutive times. Relative recovery for PAHs ranged from 73 % to 118 % and from 104 % to 106 % for deuterated PAHs. In order to avoid contamination, all laboratory instruments and non-volumetric glassware used for extraction were cleaned with highly polar organic solvents and muffled at 500 °C for 4 h, following the

Method 610 for PAHs analysis from the United States Environmental Protection Agency (US EPA, 1986). The analyzed compounds are in the supplementary data (Table S1).

Hg determination in the sediment samples was performed using the Direct Mercury Analyzer DMA-80 Tri Cell (Milestone, Sorisolev (BG), Italy). During the analysis, the lyophilized sediment samples were weighed (approximately 100 mg) and placed in nickel boats. Mercury determination using a direct mercury analyzer (DMA-80) allowed for three calibration curves, which showed the following results: 1) linear range (cell 0 = 0.010–3 ng; cell 1 = 3–10 ng; cell 2 = 10–100 ng); 2) linear regression (cell 0 = 0.1410 mHg + 0.0004; cell 1 = 0.0510 mHg + 0.0064; cell 2 = 0.00085 mHg + 0.00019); and 3) coefficient of determination (R^2) = 0.9994, 0.9991, and 0.9990, respectively. The LOD and LOQ were determined using the standard deviation (SD) values of the blank replicates following the same PAH equation. The LOD and LOQ values were 0.004 and 0.012 ng g^{-1}, respectively, and the LOD was applied. Method accuracy was realized and confirmed using certified reference materials of marine sediment and fish protein (MESS-3 and DORM-4) from the National Research Council, Canada (NRCC). The certified reference values were 0.091 ± 0.009 mg kg^{-1} and 0.410 ± 0.055 mg kg^{-1}, respectively. The results obtained by the DMA method were 0.086 ± 0.003 for sediment marine and 0.414 ± 0.003 mg kg^{-1} for fish protein. All chemical results were analyzed for differences using Student's *t*-test for the factor Beach (Dura and Boracéia) and Welch's correction, if necessary.

2.3. Different plastic pellet colors

To test the toxicity of plastic pellets of different colors, *E. armata* from the Dura Beach population was exposed to two color groups of plastic pellets (white and yellowish) and a control treatment (without pellets) using the Dura Beach sediment, following the method described in the previous sections of this manuscript. We chose the Dura Beach population because it presented the best sensitivity to the toxic effects of plastic pellets. Pellets were visually separated into two groups: the white (white, translucent, and gray tones) and the yellowish (yellow, orange, and brown tones). A total of 40 pellets of each color group were added to the surface layer of the sediment within a beaker and exposed for 6 h, as in the previous assays. Each treatment had nine replicates, and the experiment was repeated four times (108 replicates total, 36 per treatment).

A Generalized Mixed Model (GLMM) with a Poisson distribution was built and the dependent variable (mortality) was tested using two factors: Date (Factor 1, random, 4 levels), and Treatment (Factor 2, fixed, 3 levels).

3. Results

3.1. Different populations under the same exposure conditions

Fig. 2 shows the mortality of individuals of *E. armata* sampled in Dura and Boracéia beaches, exposed to plastic pellets. Treatment effect on *E. armata* populations was dependent on the beach assessed (Beach x Treatment: AIC = 203.9, X^2 = 3.9, p = 0.04), since a difference in mortality between control and pellet treatments was noted only for the Dura Beach population ($p_{bonferroni}$ = 0.03, Fig. 2). Individuals in the population from Dura Beach exposed to plastic pellets had 3.5 times higher mortality values than those in the control treatment (RR = 3.56, CI95%: 1.51–8.40). The mortality in Dura Beach was also 3.1 times higher than the Boracéia Beach population, when exposed to plastic pellets (RR = 3.12, CI95%: 1.41–6.98). There were no significant differences in mortality of control and pellets treatment to individuals from Boracéia Beach ($p_{bonferroni}$ = 1, Fig. 2). The random factor Date was also significant for both groups (p = 0.03).

Fig. 3 shows the biomarkers results for *E. aramta* from Dura Beach and Boracéia Beach populations when exposed to plastic pellets. None of the analyzed biomarkers were statistically significant for any factor,

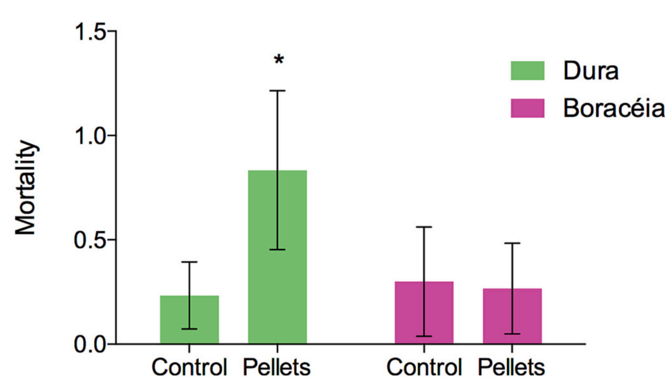

Fig. 2. Average mortality of *Excirolana armata* in raw count numbers in each treatment (control and pellets) per beach population (Dura Beach and Boracéia Beach) tested under the same conditions: exposed in Dura Beach sediment at the same time. Error bars represent a 95 % confidence interval and asterisks represent toxic effects compared to the control of the same beach population.

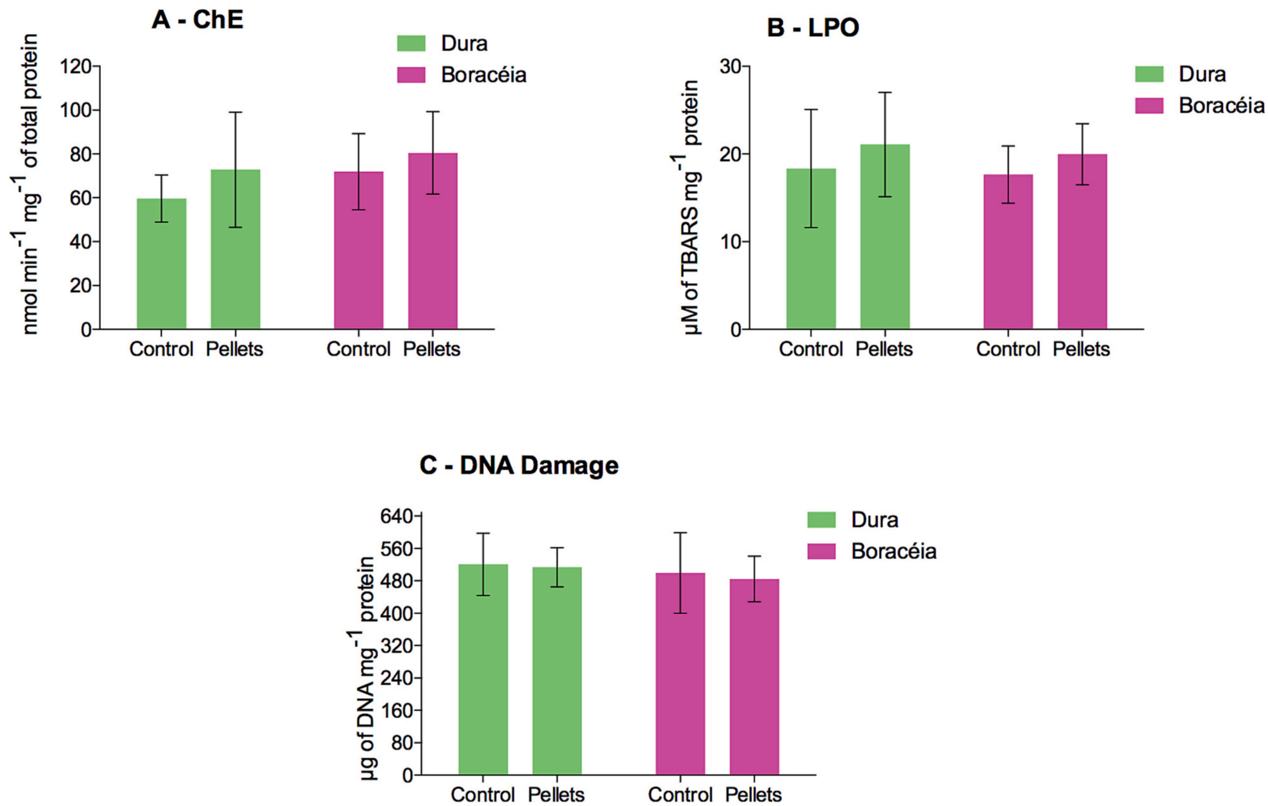

Fig. 3. Average values of cholinesterase (ChE) expressed in nmol min^{-1} mg^{-1} of total protein (A), lipid peroxidation analysis (LPO) expressed in µM TBARs mg^{-1} total protein (B) and DNA damage analysis expressed in µg DNA mg^{-1} of total protein (C) for *Excirolana armata* per treatment (Control and Pellets) and per beach population (Dura Beach and Boracéia Beach). Error bars represent 95 % confidence intervals.

including random factor (Fig. 3).

3.2. Different sediments for the same population

Conversely, we did not find any effect of pellet treatment on *E. armata* mortality when comparing the control group to the pellets exposed group (p = 0.92). We found 4 times higher mortality (RR = 4.24, CI95%: 1.16–15.18) for treatments (control and pellets) exposed to Dura Beach sediment than those exposed to Boracéia Beach sediment (AIC = 123.3, X^2 = 4.8, p = 0.02) (Fig. 4). The random factor Date was not significant (p = 0.07).

The average organic content of the sediment in Dura Beach was 0.68 % (CI95%: 53–83), while on Boracéia Beach it was 0.47 % (CI95%: 32–63). The average grain size (Φ) was 3.42 (CI95%: 3.34–3.51) at Dura beach and 2.81 ± (CI95%: 2.73–2.90) at Boracéia Beach. Dura Beach had higher OM concentration and smaller grain size (Φ) than Boracéia Beach (t = 2.02, df = 18, p = 0.059 [marginal]; t_{welch} = 10.7, df 9.2, p < 0.001; respectively). The concentrations of PAHs, N-PAHs, and O-PAHs in the sampled sediment in both beaches were below LOD for all analyzed compounds (Supplementary Material, Table S1). The average concentrations of Hg at Dura and Boracéia beaches were 2.6 ng g^{-1} (CI95%: 1.5–3.8) and 2.4 ng g^{-1} (CI95%: 1.2–3.5), respectively, with no significant difference between beaches (t = 0.45, df = 4, p = 0.67).

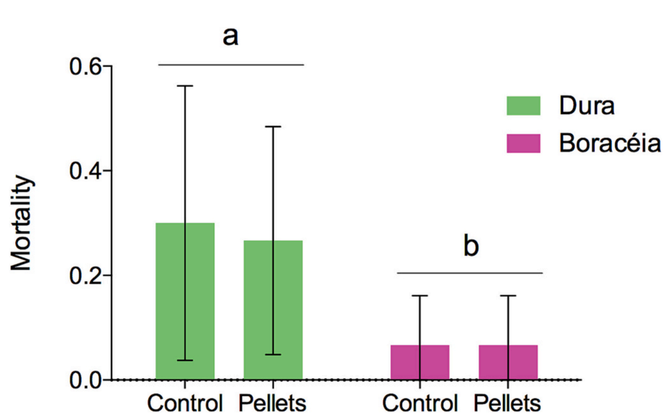

Fig. 4. Average mortality of *Excirolana armata* in raw count numbers from the Boracéia Beach population was tested for sediment from Dura Beach and Boracéia Beach. Error bars represent 95 % confidence intervals. Different letters indicate significant differences between beach sediments in terms of toxicity.

3.3. Different plastic pellet colors

We found a mortality difference between pellet treatments (AIC = 237.8, X^2 = 15.9, p < 0.001), with 2.6 times more mortality in white pellet exposure compared to the control (RR = 2.6, CI95%: 1.25–5.38), and 4 times more mortality in yellowish pellet exposure than in the control (RR = 4, CI95%: 2–7.98) (Fig. 5). There is no significant difference between color groups ($p_{bonferroni}$ = 0.25). The random factor Date was significant (p = 0.04).

4. Discussion

Different *E. armata* populations exposed to plastic pellets exhibited distinct responses. When exposed to the same sediment under the same conditions, only the population of Dura Beach showed signs of toxicity, in which pellets induced 3.1 times higher mortality (or cannibalism behavior; see Izar et al., 2022b). The Boracéia's population seemed to be resistant to plastic pellet stressors, which can be caused by the fact that this beach is closer to Santos Port (a plastic pellet source) and has a higher number of beached plastic pellets per area than Dura beach (Izar

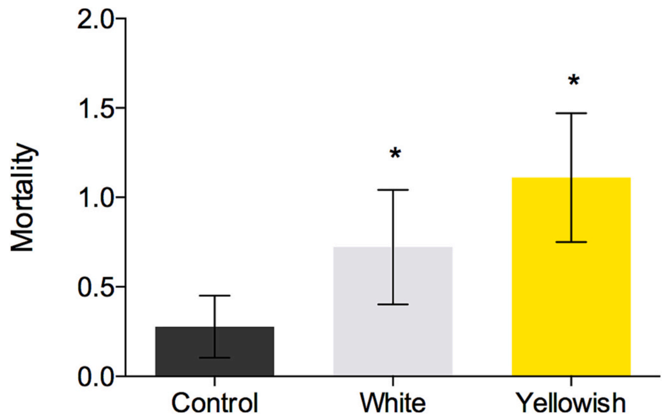

Fig. 5. Average mortality of *Excirolana armata* in raw count numbers from the Dura Beach population in Dura Beach sediment exposed to different pellet color groups (white and yellowish) and control without pellets. Error bars represent 95 % confidence intervals and asterisks represent toxic effects compared to the control.

et al., 2019). This population showed very low mortality (similar to the control values) regardless of the sediment tested (Dura Beach or Boracéia Beach sediments).

E. armata has proven to be highly tolerant to environmental and anthropogenic stressors because of its resistance to plastic pellets, as observed in the Boracéia Beach population. This isopod has already demonstrated some degree of resistance to abrupt salinity variations (Lozoya and Defeo, 2006; Lozoya et al., 2010; Fanini et al., 2017; Laurino and Turra, 2021), anthropogenic erosion (Thompson and Sánchez de Bock, 2007), and to non-ingested plastic pellets exposure. High mortality in the Dura Beach population exposed to plastic pellets and their changes in vertical distribution to avoid floods with different salinities (Laurino et al., 2020) suggest that this resistance selection is possibly acquired over generations when exposed to a new (unfamiliar) stressor. This resistance can be behavioral, avoiding the stressor, as is the case with changes in salinity (Laurino et al., 2020), or physiological. The resistance observed in this study seems to be caused by this last case. Physiological adaptations have already been described for mosquitoes (Nkya et al., 2013), and are related to their fast life cycle. Similar to mosquitoes, isopods are arthropods with a fast life cycle, suggesting that this resistance can be acquired in a short time (Nkya et al., 2013). Nonetheless, we did not test this possible resistance heritability here, a hypothesis that must be explored in future studies.

Interestingly, no sublethal effects were found in either isopod population when exposed to plastic pellets. This is unlike the pattern of increasing LPO and DNA damage levels caused by microplastic exposure found by Prokić et al. (2019), however follows the pattern found in our previous study (Izar et al., 2022b). The results of our studies may be associated with behavioral changes when this species is exposed to plastic pellets and does not reach physiological stress. The same values for all biomarkers could represent a sublethal effect at the same intensity with or without pellets exposure, corroborating with the idea of behavioral changes rather than lethal effects such as mortality.

The population of Boracéia Beach responded differently when tested in different sediments. This population showed approximately 4 times more intense response when exposed to Dura Beach sediment in both treatments (control and pellets). Therefore, the characteristics of the sediment should be considered. Dura Beach sediment grains are finer than those of Boracéia Beach sediment. These characteristics are favorable for *E. armata*, once this species shows a preference for and increased abundance in fine sands (Defeo et al., 1997; Lozoya et al., 2010; Fanini et al., 2017). In addition, OM might also be considered when evaluating toxicity. It can be an important route of contamination, facilitating the bioavailability of some contaminants from plastic pellets (Ferraz et al., 2020). For *E armata*, this is an important factor due to its higher abundance and preference for sediments with low levels of OM (Lozoya et al., 2010), as those from Boracéia Beach. Even with no levels of PAHs contamination and similar and very low Hg contamination on both beaches, other contaminants not tested may be present in the beach sediment. Pharmaceuticals are emerging pollutants that have proven to be toxic when associated with plastics (Nobre et al., 2020 and 2022). Ammonia may also be considered because high toxicity can be associated with its presence in the sediment (Araujo et al., 2013; Campos et al., 2016). Both may be present in high concentrations at Dura Beach because of the elevated sewage input (CETESB, 2019; Nobre et al., 2020). The proximity of mangroves to the beach can also be a natural source of ammonia in the region. However, for the isopod population from Boracéia Beach, Dura Beach sediment induced four times more toxicity with or without pellet exposure than when exposed to Boracéia Beach sediment. The non-toxic effect of plastic pellets on this population reinforces the importance of considering the characteristics of the sediment as a factor for toxicity. On the other hand, there was no difference in PAHs or mercury contamination in beach sediments between the two beaches, contrary to what was found for marine sediments and sewage, in which sediments from the Dura Beach area were more contaminated than those from Boracéia Beach (CETESB, 2019; CETESB, 2020b; Moreira et al., 2021).

All chemicals analyzed were below Level 1 (low probability of adverse effects to biota) of the Brazilian Environmental Council (CONAMA 454/2012), on both beaches. The same chemical contamination level (PAHs and Hg) in the sediments of the studied beaches does not completely refute our hypothesis of multiple stressors. However, OM may act as a route for the bioavailability of other contaminants not tested carried by plastic pellets to sandy beaches. In our first experimental results (different populations in the same exposure conditions), indicate the toxicity found for the Dura Beach population when exposed to plastic pellets, unlike the Boracéia Beach population (both tested in Dura Beach sediment), suggests toxicity for multiple stressors. Toxicity in the Dura Beach sediment to the *E. armata* population from Boracéia Beach, with or without exposure to plastic pellets, reinforces this hypothesis. The higher level of OM in the Dura Beach sediment can also increase the bioavailability of contaminants to the benthic macroinvertebrate community and might be a persistent and natural stressor in the local isopod population. Ammonia can also present as a natural stressor, especially in Dura beach due the proximity of a mangrove. When these isopods are exposed to an additional stressor (plastic pellets carrying more contaminants that are bioavailable by OM), they reach a stress threshold, resulting in mortality (cannibalism behavior). Similar results were found by Maulvault et al. (2019), in which stress on juvenile fish was more severe when the three stressors acted simultaneously.

In all experiments, mortality of *E. armata* was observed as a result of intraspecific interactions such as necrophagy and cannibalism, corroborating Izar et al. (2022b). In both studies, only living individuals were found inside beakers after the exposure time, with no signs of dead individuals. The presence of plastic pellets intensifies the agonistic interactions of this species (Bergamino et al., 2011; Bergamino et al., 2012), in which living individuals attack dead or dying individuals. Mortality (cannibalism) was more evident in the population of Dura Beach, although it was also observed in the population of Boracéia Beach.

Beach-stranded plastic pellets were toxic in all exposed combinations: mixed, regardless of color, and separated by color (white and yellowish). When separated, pellets of both colors were toxic, with yellowish tones tending to cause higher toxicity than white tones. Beach-stranded plastic pellets tend to concentrate more contaminants in the darkest tones of the yellow (Endo et al., 2005; Fotopoulou and Karapanagioti, 2012). For example, Yamashita et al. (2018) observed polychlorinated biphenyl (PCB) concentrations up to 29 times higher in orange and brown than in white plastic pellets. The toxicity of white pellets is related to industrial additives that tend to be volatile (fast biodegradation) and they are easily released when a virgin pellet reaches the environment (Teuten et al., 2009). Therefore, according to our data, corroborating with Gandara e Silva et al. (2016), industrial additives seem to be less concentrated and therefore less toxic than hydrophobic contaminants adsorbed, which are present in higher concentrations in beach-stranded plastic pellets. Unlike the results of Nobre et al. (2015) and Izar et al. (2019), which showed higher toxicity of virgin plastic pellets to sea urchin larvae and no difference in toxicity between white and colored beach-stranded plastic pellets, we found toxicity in both color groups. This difference in toxicity might be related to the exposure method, since in Nobre et al. (2015), sea urchin larvae were exposed to leachate from plastic pellets, in which virgin pellets tend to desorb more volatile chemical compounds (industrial additives). In contrast, in the Izar et al. (2019) assays, the density of the pellets tested was extremely high, probably reaching the stress limit of the organisms tested in both pellet colors (white and colored). In addition, both studies used embryos in their exposures, which tend to be a sensitive stage in the life cycle of organisms, prone to toxicity, and masking small differences in toxicity. Our study was performed using adults of a very resistant species, and this natural resistance might have highlighted the subtle difference in toxicity between pellet colors because resistant species take longer to reach their toxicological stress limit.

5. Conclusion

Organisms with a fast life cycle can acquire resistance to stressors and potentially mask their toxic effects. We encourage further studies in this regard. Plastic pellets can act as carriers of hydrophobic contaminants to sandy beaches, and these contaminants can become bioavailable to sandy beach macrobenthic populations through organic matter. Plastic pellets should also be considered a multi-stressor toxicity matrix. We observed an increase in *E. armata* necrophagy and cannibalism when exposed to a new stressor (plastic pellets). To avoid misunderstanding or masking toxicity results, we encourage the consideration of intraspecific and interspecific interactions in ecotoxicological assays. Finally, pellet color is relevant as a factor for toxicity, with yellowish colors inducing higher toxic effects than white ones. Hydrophobic contaminants adsorbed in plastic pellets are more toxic to macrobenthic populations because of their high concentrations in beach-stranded plastic pellets.

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CRediT authorship contribution statement

G.M. Izar: Conceptualization, Methodology, Formal analysis, Writing – original draft, Investigation, Visualization, Project administration. **T.Y. Tan:** Conceptualization, Methodology, Investigation, Writing – review & editing. **I.R.A. Laurino:** Investigation, Writing – review & editing. **C.R. Nobre:** Investigation, Writing – review & editing. **M.P.M. Vivas:** Methodology, Investigation, Writing – review & editing. **P.K. Gusso-Choueri:** Investigation. **C.S.A. Felix:** Investigation, Writing – review & editing. **B.B. Moreno:** Investigation. **D.M.S. Abessa:** Writing – review & editing, Funding acquisition, Resources. **J.B. de Andrade:** Resources. **S.T. Martinez:** Writing – review & editing. **G.O. da Rocha:** Writing – review & editing, Funding acquisition, Supervision, Resources. **A.C.R. Albergaria-Barbosa:** Writing – review & editing, Funding acquisition, Supervision, Resources.

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.

Data availability

Data will be made available on request.

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