Laboratoire d'Océanologie et de Géosciences
UMR 8187 – CNRS – Univ. Lille – ULCO

THÈSE DE DOCTORAT

Pour obtenir le grade de docteur de l'Université de Lille

École doctorale Science de la Matière, du Rayonnement et de l'Environnement
Spécialité : Géosciences, Écologie, Paléontologie, Océanographie

Par Camille DELAETER

Impact des lixiviats de bioplastiques et plastiques conventionnels sur les organismes benthiques intertidaux : une approche comportementale

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Impact of leachates from bioplastics and conventional plastics on intertidal benthic organisms: a behavioral approach

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Soutenue publiquement le 7 Décembre 2023 devant un jury composé de

Alex FORD, Professeur, University of Portsmouth (Angleterre)	Rapporteur - Président du jury
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Dannielle GREEN, Professeur Associée, Anglia Ruskin University (Angleterre)	Examinatrice
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Storms make trees take deeper roots – Dolly Parton

SCIENTIFIC CONTRIBUTION DURING MY PHD THESIS

PUBLICATIONS FROM THIS PHD THESIS

Delaeter, C., Spilmont, N., Delleuze, M., & Seuront, L. (2023). Lack of behavioral effect of surgical mask leachate on the Asian shore crab *Hemigrapsus sanguineus*: Implications for invasion success in polluted coastal waters. Science of The Total Environment, *892*, 164683.

Delaeter, C., Seuront, L., Bouchet, V.M.P., & Spilmont, N. (2022). Plastic leachates: Bridging the gap between a conspicuous pollution and its pernicious effects on marine life. Science of The Total Environment, *826*, 154091.

OTHER PUBLICATIONS

Seuront, L., Zardi, G.I., Uguen, M., Bouchet, V.M.P, **Delaeter, C.**, Henry, S., Spilmont, N., & Nicastro, K.R. (2022). A whale of a plastic tale: A plea for interdisciplinary studies to tackle micro- and nanoplastic pollution in the marine realm. Science of The Total Environment, *846*, 157187.

Seuront, L., **Delaeter, C.**, Henry, S., Nicastro, K.R., Spilmont, N., Uguen, M., & Zardi, G.I., (sous presse). Un océan de plastique : historique, chiffres, impacts et 'solutions'. In : Schmitt FG & Brisabois A (eds.), La Manche orientale : vue interdisciplinaire. Presses Universitaires du Septentrion.

ORAL PRESENTATION

Delaeter, C., Spilmont, N., Bouchet, V.M.P., & Seuront, L. Les lixiviats de plastique : la face cachée d'une pollution en constante augmentation. MARCO conference, Boulogne-sur-mer, France, October 13 – 15th 2021

POSTERS

Delaeter, C., Seuront, L., Delleuze, M., Bouchet, V.M.P., & Spilmont, N**.** The potential chemical impact of plastic leachates on the scototactic and thigmotactic behaviors of the invasive Asian shore crab *Hemigrapsus sanguineus*. ICAIS conference, Oostende, Belgium, April 18 – 22nd 2022.

Delaeter, C., Spilmont, N., Bouchet, V.M.P., & Seuront, L. Le potentiel impact des lixiviats de microplastiques sur deux espèces benthiques : le crabe Hemigrapsus sanguineus et le foraminifère Haynesina germanica. GDR Polymères & Ocean, Brest, France, June 30th – July 1st 2022.

TEACHING

During the first two years of my PhD thesis, I have taught to Bachelor and Master students at the University of Lille (128 h in total):

- tutorial and practical classes of Biostatistics

- tutorial classes of Ecosystemic Ecology

- tutorial classes of Tools in Ecosystems and Population Genetics

- practical classes in Marine Ecosystems

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surrounded by matching color walls, which respectively allow to assess the preference for dark vs. white areas (i.e. scototaxis) and the response to a physical barrier (i.e. thigmotaxis). The 'acclimatization cage' is placed on the grey central square of the experimental arena and allow to assess the startle response.

Figure 2. Schematic illustration of (A) the behavioral variables considered for 10 minutes record of the anxiety behaviors of *Hemigrapsus sanguineus*, as (1) the startle response, (2) the scototaxis, (3) the thigmotaxis, (4) the level of activity (arrows represent active individuals) and (5) the vigilance; and (B) the classification of the observed thigmotactic behavior, as walling (i.e. preference for dark or light areas; a), cornering (i.e. preference for moving toward or away from physical barriers; b) and climbing (c).

Figure 3. Anxiety behaviors of *Hemigrapsus sanguineus* after the 6h-exposure to virgin surgical mask's leachate (1 mask/L at different desorption time) . The figures represent the average time spent (a) in the central grey square once the cage is lifted (s), i.e. startle time, (b) in contact with discontinuities once the central grey square is left (%), i.e. thigmotaxis, (c) in black areas once the central grey square is left (%), i.e. scototaxis, and (d) with eyes directed toward the wall once when walling and cornering (%), i.e. vigilance, during the 10 min-record depending on the mask's desorption time (h). "0" is the control treatment and corresponds to the basic behaviors as described in 3.1. Errors bars are standard errors, n is the number of analyzed individuals for each treatment (identical for each panel) and b is the number of bold individuals.

Figure 4. Percentage of the time *Hemigrapsus sanguineus* spent being either active or inactive during the 10 min-record in black or white areas, after 6h-exposure to virgin surgical mask's leachate (1 mask/L); shown as a function of surgical mask incubation time (h). "0" is the control treatment and corresponds to the basic behaviors as described in 3.1. Errors bars are standard errors.

Table 1. Variability in the anxiety behavior of *Hemigrapsus sanguineus* exposed to natural seawater (i.e. "0") or solutions of mask leachate (1 mask/L) desorbed during 6 to 96 h assessed through the coefficient of variation (%) of the startle time (time spent in grey once the cage is lifted), the scototactic response (time spent in black areas once the central grey square is left), the thigmotactic response (time spent in contact with a discontinuity once the central grey square is left) and the vigilance response (time spent with eyes' direction toward the center of the arena while walling or cornering) of "0" is the control treatment. The record of behaviors lasted 10 minutes.

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CHAPTER III

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Figure 2. Anxiety behaviors of *Hemigrapsus sanguineus* after the 6 h-exposure to plastic pellet's leachate (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid; 24 h desorption; black bars). The figures represent the average time spent (A) in the central grey circle once the cage is lifted (s), i.e. startle time, (B) in contact with discontinuities once the central grey circle is left (%), i.e. thigmotaxis, and (C) with eyes directed toward the center of the arena when walling (%), i.e. vigilance, during the 10 min-record depending on the polymer type. "C" is the control group (grey bars). Errors bars are standard deviation, n is the number of analyzed individuals for each treatment (identical for each panel) and b is the number of bold individuals.

Figure 3. Scototactic behaviors of *Hemigrasus sanguineus* observed during the 10 min-record after a 6 h-exposure to the leachates of plastic pellets (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid; 24 h desorption; black bars). The figures represent (A) the average time spent in black areas (%), i.e. scototaxis, (B) the number of individuals observed to enter a black area first after leaving the central grey circle (%) and (C) the number of individuals observed to first touch a black wall (%). "C" is the control group (grey bars). Errors bars are standard deviation and letters above the bars represent significant differences between treatments (Chi^2 test).

Figure 4. Percentage of the time *Hemigrapsus sanguineus* spent being either active or inactive in black or white areas, after 6 h-exposure to plastic pellets' leachates (24 h desorption); shown as a function of the polymer type (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid). "C" is the control treatment. Significant difference between treatments for each activity level between treatments is represented by a star (Kruskal-Wallis tests).

Table 1. List of additives found in the pellets of different polymer depending on their function. Abbreviations means: tributyl Acetyl Citrate (ATBC), benzyl butyl phthalate (BBP), 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE153), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE154), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE183), butylated hydroxytoluene (BHT), bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), di-allyl phthalate (DAIP), phthalates dibutyl phthalate (DBP), bis-2-ethylhexyl adipate (DEHA), di(2-ethyhexyl)phthalate (DEHP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), diisodecyl phthalate (DIDP), diisoheptyl phthalate (DIHP), dimethyl phthalate (DMP), nonylphenol monoethoxylate (NP1EO),

nonylphenol (NPs), tributyl phosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCPP).

Table 2. Variability in the anxiety behavior of *Hemigrapsus sanguineus* exposed to natural seawater (i.e. “C”) or solutions of plastic pellet leachates (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid) desorbed during 24 h assessed through the coefficient of variation (%) of the startle time (time spent in grey once the cage is lifted), the scototactic response (time spent in black areas once the central grey square is left), the thigmotactic response (time spent in contact with a discontinuity once the central grey square is left) and the vigilance response (time spent with eyes’ direction toward the center of the arena while walling or cornering). The record of behaviors lasted 10 minutes.

CHAPTER IV

Figure 1. Boxplot of the total distance moved (mm) by *Haynesina germanica* during 20h-exposure to a low or high concentration of plastic pellet leachates from different polymer type. BP is beached pellets, PA is virgin polyamide pellets, PE is virgin polyethylene pellets. Potential significant differences are represented by letters above the boxes, different letters mean significant differences, each experimental session being analyzed independently from one another.

Figure 2. Speed (mm/h) of *Haynesina germanica* during the initial 10 minutes step when exposed to virgin polyethylene (PE), virgin polyamide (PA) and beached pellets (BP) leachates. Potential significant differences are represented by letters above the boxes, different letters mean significant differences, each experimental session being analyzed independently from one another.

Figure 3. Temporal dynamic of the mean speed of *Haynesina germanica* when exposed to virgin polyethylene (PE), virgin polyamide (PA) and beached pellets (BP) leachates. Each graph represents the experimental distribution of the mean speed (calculated at each time step, i.e. 10 min, on the 30 individuals; dots) and the modelized distribution using the Pratt et al. (1980) photosynthesis – irradiance equation (line) of the control group (green), the low concentration (BP_{low}, PA_{low} and PE_{low}; orange) and the high concentration (BP_{high}, PA_{high} and PE_{high}; red).

Figure 4. Boxplots representing the NGDR of the trajectories of *Haynesina germanica* during a 20h-exposure to a low (10) or high (50) concentration of plastic pellet leachates from different polymer types. PE is virgin polyethylene, PA is virgin polyamide, BP is beached pellet. Potential significant differences are represented by letters above the boxes, different letters mean significant differences, each experimental session being analyzed independently from one another.

Table 1. List of additives found in the pellets of different polymer depending on their function. Abbreviations means: tributyl Acetyl Citrate (ATBC), benzyl butyl phthalate (BBP), 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE153), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE154), 2,2',3,4,4',5',6-heptabromodiphenyl ether

(BDE183), butylated hydroxytoluene (BHT), bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), di-allyl phthalate (DAIP), phthalates dibutyl phthalate (DBP), bis-2-ethylhexyl adipate (DEHA), di(2-ethyhexyl)phthalate (DEHP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), diisodecyl phthalate (DIDP), diisoheptyl phthalate (DIHP), dimethyl phthalate (DMP), nonylphenol monoethoxylate (NP1EO), nonylphenol (NPs), tributyl phosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCPP).

Table 2. Level of activity of foraminifera exposed to virgin polyethylene (PE), virgin polyamide (PA) and beached pellets (BP) leachates assessed through the activity index. The mean (± standard deviation) and the minimal and maximal value are given for each treatment; low and high concentrations and their control (C). The significant differences between the leachate treatments and their respective control are represented by an asterisk (*).

Table 3. Parameters of the Pratt et al. (1980) equation (α, *Vmax* and β) and the coefficient of determination of the modelized temporal dynamic of speed of *Haynesina germanica* exposed to beached pellet (BP), polyamide (PA) and polyethylene (PE) leachates at low and high concentrations and their respective control (C). Units are mm/h for α and β, and mm/h^2 for *Vmax*.

Table 4. Summary of the significant differences observed in the motion behavior of *Haynesina germanica* exposed to the leachates of polyethylene (PE), polyamide (PA) and beached pellets (BP).

Figure S1. Additive content of virgin polyethylene pellets (A), virgin polyamide pellets (B) and beached pellets (C) (mean ± standard deviation; n = 3). For acronym interpretation, refer to Table 2. Note the two-orders of magnitude difference in additive concentrations observed between polyethylene pellets and both polyamide and beached pellets.

CHAPTER V

Box 1. The different types of cirral behaviors

Figure 1. Representation of the experimental procedures.

Table 1. List of additives found in the pellets of different polymer depending on their function. Abbreviations means: tributyl Acetyl Citrate (ATBC), benzyl butyl phthalate (BBP), 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE153), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE154), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE183), butylated hydroxytoluene (BHT), bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), di-allyl phthalate (DAIP), phthalates dibutyl phthalate (DBP), bis-2-ethylhexyl adipate (DEHA), di(2-ethyhexyl)phthalate (DEHP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), diisodecyl phthalate (DIDP), diisoheptyl phthalate (DIHP), dimethyl phthalate (DMP), nonylphenol monoethoxylate (NP1EO),

nonylphenol (NPs), tributyl phosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCPP).

Figure 2. Impact of plastic leachates from polyethylene (PE), polyamide (PA), beached pellet (BP) and polylactic acid (PLA) on the cirral activity (a&c) and the cirral beat frequency (CBF; b&d) when the barnacles are exposed to plastic leachate solutions desorbed at 10 (a&b) and 20 °C (c&d). (C) is the control group and the letters illustrate significant differences (Dunn tests).

GENERAL DISCUSSION & CONCLUSION

Figure 1. Graphical representation of the behavioral plasticity and individual personality.

Box 2. Tests of behavioral variability

LIST OF ABBREVIATIONS

ATBC: Tributyl acetyl citrate
ATR: Attenuated total reflectance
BBP: Butyl benzyl phthalate
BDE153: 2,2',4,4',5,5'-hexabromodiphenyl ether
BDE154: 2,2',4,4',5,6'-hexabromodiphenyl ether
BDE183: 2,2',3,4,4',5',6-heptabromodiphenyl ether
BHT: Butylated hydroxytoluene
BPA: Bisphenol A
BPF: Bisphenol F
BPS: Bisphenol S
CBF: Cirral beat frequency
CTR: Car tire rubber
CV: Coefficient of variation
DAIP: Di-allyl phthalate
DBP: Dibutyl phtalate
DDT: Dichlorodiphenyltrichloroethane
DEHA: Bis(2-ethylhexyl) adipate
DEHP: Bis(2-ethylhexyl) phthalate
DEP: Diethyl phthalate
DIBP: Di-isobutyl phthalate
DIDP: Diisodecyl phthalate
DIHP: Diisoheptyl phthalate
DMP: Dimethyl phthalate
DNA: Desoxyribonucleic acid
FTIR: Fourier-transform infrared
HDPE: High density polyethylene
KW: Kruskal-Wallis
LDPE: Low density polyethylene
NP: Nonylphenol
NP1EO: Nonylphenol monoethoxylate
PA: Polyamide
PAH: Polycyclic aromatic hydrocarbon
PAM: Polyacrylamide
PAN: Polyacrylonitrile
PBDE: Polybrominated diphenyl ethers
PC: Polycarbonate
PCB: Polychlorinated biphenyls
PE: Polyethylene
PES: Polyester
PET: Polyethylene terephthalate
PFA: Phenol-formaldehyde
PIR: Polyisopropene rubber

PLA: Polylactic acide
PMMA: Polymethyl methacrylate
POP: Persistent organic pollutant
PP: Polypropylene
PP&A: Polyesters, polyamide & acrylics
PS: Polystyrene
PUR: Polyurethane
PVA: Polyvinyl acetate
PVC: Polyvinyl chloride
ROS: Reative oxygen species
SBR: Styrene-butadiene rubber
TBP: Tributyl phosphate
TCEP: Tris(2-chloroethyl)phosphate
TCPP: Tris(2-chloroisopropyl)phosphate
TDCPP: Tris(1,3-dichloro-2-propyl)phosphate
TWP: Tire wear particles
WMW: Wilcoxon-Mann-Whitney

Note on the Chapter I

Please note that in the first chapter polyamide is abbreviated PLA. In the rest of the manuscript, PLA and PA are the abbreviations of polylactic acid and polyamide, respectively.

GENERAL INTRODUCTION

Behavior encompasses the diverse actions undertaken by animals to interact with, respond to and influence their environment (Mench, 1998). Behavior is even described as the organism's "first line of defense" (Mench, 1998) in response to the intrinsically changing environment (e.g. tidal, diel and seasonal cycles). Individual behavior is intricately shaped by various genetic, morphologic and physiological processes (i.e. metabolic, endocrine, neurological and sensorial; Scott and Sloman, 2004; Sih et al., 2010). In the ever-changing environment, when an individual perceives a stimulus through its sensory system, this information is subsequently transduced into a neuronal or hormonal response that ultimately leads to behavioral responses (Wingflied, 2013; Fig. 1). The manifestation of a specific behavior is contingent upon factors such as the individual's age, sex, physical condition and life history (Bolnick et al., 2011). The ability to adapt and adjust behavior in accordance with environmental shifts empower the organism to maximize its overall fitness (Candolin and Wong, 2019; Saaristo et al., 2018).

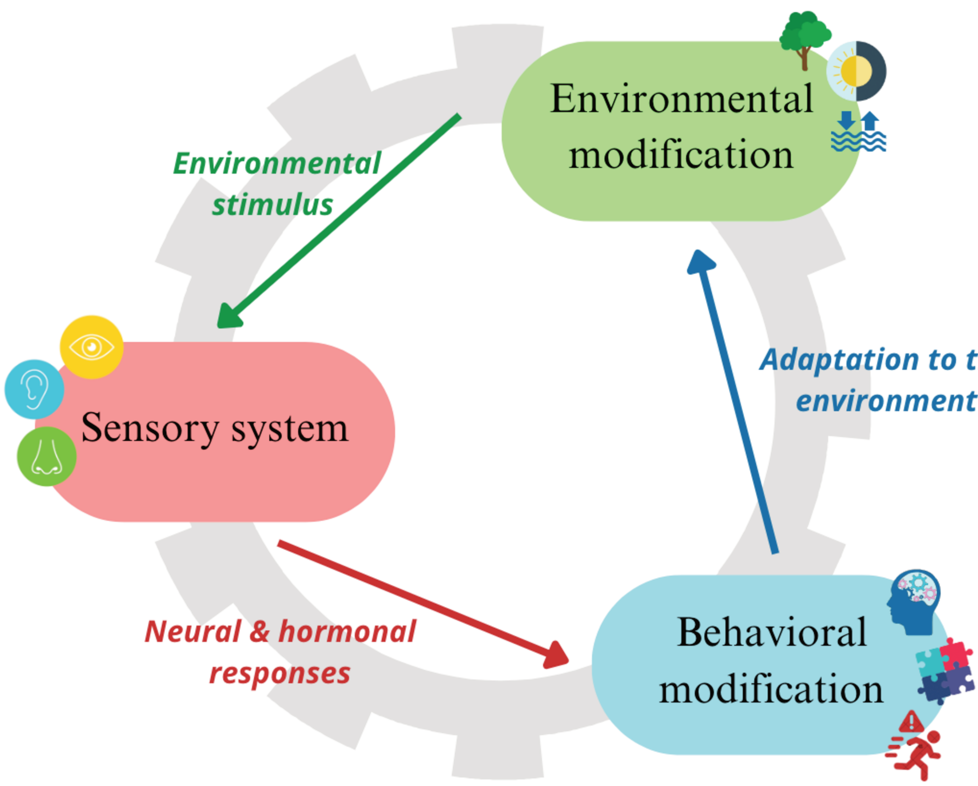


Figure 1. Schematic representation of the behavioral modification induced by environmental changing.

At the individual scale, adapting behavior to the environment enables, for example, preys to flight when perceiving predator scent, intertidal organisms to move toward dark areas to avoid desiccation at low tide or migrating animals to travel hundreds of kilometers when the season is changing. Nevertheless, the impact of these behaviors extends far beyond the individual level. Through interactions between conspecifics and other species, individual behavior plays a pivotal role in shaping various ecological processes that, in turn, influence organisms' abundance, diversity but also survival and extinction at the population and community levels (Sih et al., 2011, 2010; Fig. 2). By shaping population and community dynamics, individual behavior ultimately exerts a significant impact on ecosystem functions (Candolin and Rahman, 2023; Candolin and Wong, 2019; Saaristo et al., 2018; Wong and Candolin, 2015; Fig. 2). Individual's behaviors is also molded by historical experiences, and as such play an important role in the mechanisms of natural selection and evolutionary processes (Duckworth, 2009; Fig. 2). Therefore, behavior emerges as an essential parameter that forges the connection between individuals, ecosystem processes and evolution.

Nowadays, behavioral responses to environmental changes face unprecedented challenges due to the rapid and detrimental nature of alterations linked to anthropogenic influences. On one hand, events such as urbanization, habitat destruction, globalization, increase in natural catastrophes and global warming, introduce tremendous hurdles to cope with through behavioral adaptations (Sih et al., 2011). Furthermore, human activities shoulder significant responsibility for environmental contamination (Arnold et al., 2014), inducing drastic alterations in behaviors (Brodin et al., 2014; Saaristo et al., 2018; White and Briffa, 2017; Zala and Penn, 2004), which may lead to organism's failure to effectively cope with its surroundings. For example, in response to a predator stimulus, a typical behavior would entail a flight response (Briffa and Sneddon, 2007; Kalueff et al., 2013; Maximino et al., 2010b). However, under conditions of contamination (e.g. by the range of chemical compounds found in the environment), a prey is likely to struggle to perceive the stimuli and thus fail to escape the predator (Briffa and Sneddon, 2007). Contaminants thereby jeopardize the organism's 'first line of defense' by instigating maladaptive behaviors or even inhibiting any behavioral response. Contaminants have the potential to disrupt an array of behaviors: from reproductive activities (e.g. difficulties attracting mates, impaired offspring care) to animal movement (e.g. reduced foraging efficiency affecting food or mate encounter) and interactions with other organisms (e.g. competition, predation; Saaristo et al., 2018). Such impairments carry direct consequences for individual fitness but, given the central role of behavior, may also have far-reaching consequences in the ecosystem functioning (Candolin and Rahman, 2023; Candolin and Wong, 2019; Saaristo et al., 2018; Wong and Candolin, 2015).

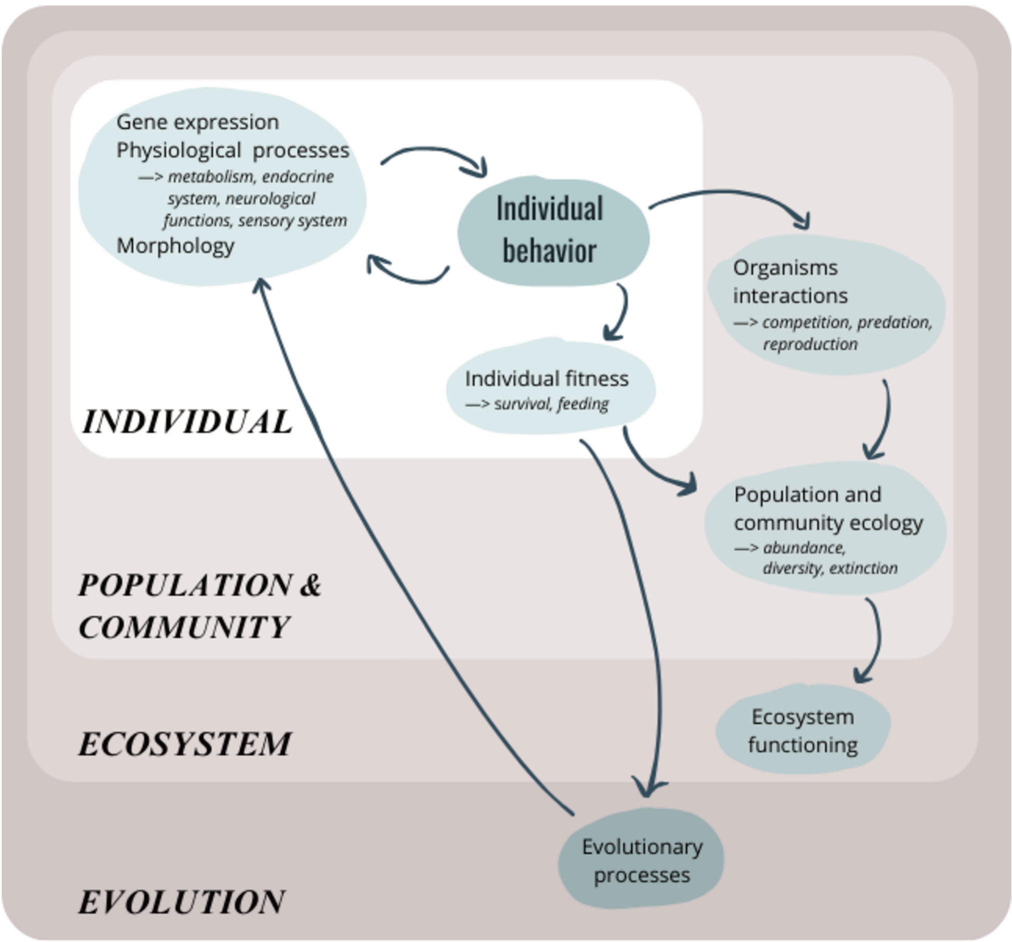


Figure 2. Illustration of the individual behavior as a central process linking morphological and physiological processes to ecosystem and evolutionary processes.

One of the most pressing forms of human-induced pollution in the modern era is plastic (Zalasiewicz et al., 2016). Derived from fossil fuels, plastics have significantly enhanced our daily lives due to their adaptable manufacturing process, low density, robustness, versatility, cost-effectiveness, decay-proof nature and resilience against corrosion (MacArthur, 2017). Plastic products have been widely used in various fields, such as packaging, construction, automotive, electronics and many other industries (PlasticsEurope, 2022). Its production has surged over the years, leading to an alarming accumulation in both landfills and natural environment (Geyer et al., 2017; PlasticsEurope, 2012; 2022). Marine environments, in particular, are heavily impacted by plastic pollution, with plastic debris comprising a substantial portion of litter on shorelines and seafloors (i.e. 50 to 90 %; Agamuthu et al., 2019). The situation may even get worse as every year 4 to 12 million tons of plastic enter the marine environment, a figure expected to increase by an order of magnitude by 2050 (Jambeck et al., 2015).

Intertidal environments, due to its close (and ever increasing) proximity with urbanized areas, are particularly prone to accumulate plastic debris originated from land via inland waterways and wastewater outflows (Mendes et al., 2021). These environments are, however, ecologically crucial both for animals' survival and ecosystem functioning (Barbier et al., 2011; Levin et al., 2001; Sardá et al., 1998). Indeed, intertidal environments provide an important abundance of prey (such as copepods, nematodes, annelids, mollusks or crustaceans) and act as a food reservoir for numerous species (Sardá et al., 1998), including humans (Barbier et al., 2011). The complex structure of these areas also serves as crucial habitat for both coastal and deep-sea organisms, acting as spawning, nesting and nursery sites and adult habitat, providing both shelter and food in early life and adult stages (Levin et al., 2001). In terms of ecosystem functioning, the intertidal zone is among the most important contributors to primary and secondary production and play a crucial role in decomposition, particle flow and nutrient recycling and transfer (Levin et al., 2001). Knowing that up to 95 % of the coastal litter are composed of plastic and given the importance of intertidal environment (Galgani et al., 2015), it is of major concern to investigate the impact of plastic on the sustainability of intertidal ecosystems under this ever-increasing pollution.

Plastic mass production starting in the 1950s, physical damages of plastic pollution has been rapidly recorded, as the earliest accounts of plastic debris in seabirds dated from the 1960s (Kenyon and Kridler, 1969). Nowadays, encounter of plastic debris with marine fauna concern 693 species in the literature—undoubtably more in reality—among which 17% are listed as near threatened, vulnerable, endangered or critically endangered in the IUCN Red List (Gall and Thompson, 2015). While the physical impacts of plastic pollution are well documented (i.e. ingestion, entanglements, habitat destruction; Andrady, 2011; Gall and Thompson, 2015; Uhrin et al., 2014), plastics also pose intrinsic harm due to the release of hazardous chemicals. Indeed, plastic are manufactured with additives that are not chemically bound to the polymer matrix and are easily leached from plastic debris to the marine environment (Hahladakis et al., 2018; Hermabessiere et al., 2017). In addition, plastic debris are prone to accumulate hydrophobic contaminants from the environment, such as persistent organic pollutants (Fries and Zarfl, 2012; Kedzierski et al., 2018; Ogata et al., 2009) at concentrations far exceeding those in the surrounding water (Hermabessiere et al., 2017). Plastic debris thus readily leach a hazardous cocktail of chemicals throughout their lifespan. These factors make plastic pollution a significant threat, as plastic leachates have been linked to carcinogenic, mutagenic and endocrine-disrupting effects (Weis, 2019), impacting survival, reproduction, embryonic development, cellular integrity, metabolism and behaviors in marine organisms; see Chapter 1 for a review (Delaeter et al., 2022).

Despite their critical role in connecting individual organisms to ecosystem functioning and their importance in adapting with environmental shifts (Candolin and Rahman, 2023; Candolin and Wong, 2019; Saaristo et al., 2018; Wong and Candolin, 2015), behaviors receive disproportionately limited attention in comparisons to other variables. Astonishingly, there have been only three studies dedicated to this critical aspect (Delaeter et al., 2022). This discrepancy is particularly noteworthy given that behavior exhibit strong sensitivity to contamination, often surpassing that of conventionnal toxicological parameters such as LC50 (Arnold et al., 2014; Little and Finger, 1990; Sih et al., 2010). Given the ever-increasing amount of plastic and their associated chemicals, it becomes imperative to identify and understand behavioral shifts as part of assessing the ecological risks of human-induced plastic pollution on the marine environment.

Objectives of the thesis:

In light of the aforementioned context, the objectives of this thesis were:

(1) To conduct an extensive review of the existing literature on plastic leachates, with a particular focus on identifying gaps in research related to methodology, polymers and species. This work has also the objective to guide the experimental choices conducting in the experiments made during the thesis

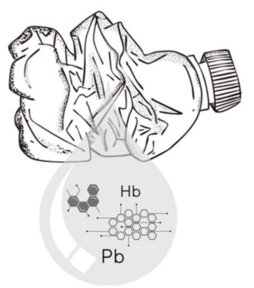
(2) To investigate the behavioral consequences of plastic leachates derived from polymers that have been previously overlooked in the literature in comparison to their presence in the environment on the behavior of key benthic species critical to intertidal environment functioning that are missing in the literature:

- The Asian shore crab *Hemigrapsus sanguineus*
- The benthic foraminifera *Haynesina germanica*
- The barnacle *Austrominus modestus*

(3) To identify suitable behavioral parameters for studying the impact of plastic leachates at individual and population scale, and to gain a deeper understanding of their potential cascading effects at higher levels of ecological organization.

(4) To make insights into the ‘plastic pollution solution’ that are bio-plastics, by evaluating their impact on the behaviors of *Hemigrapsus sanguineus* and *Austrominus modestus*, in comparison with traditional polymer.

CHAPTER I



PLASTIC LEACHATES: BRIDGING THE GAP BETWEEN A CONSPICUOUS POLLUTION AND ITS PERNICIOUS EFFECTS ON MARINE LIFE

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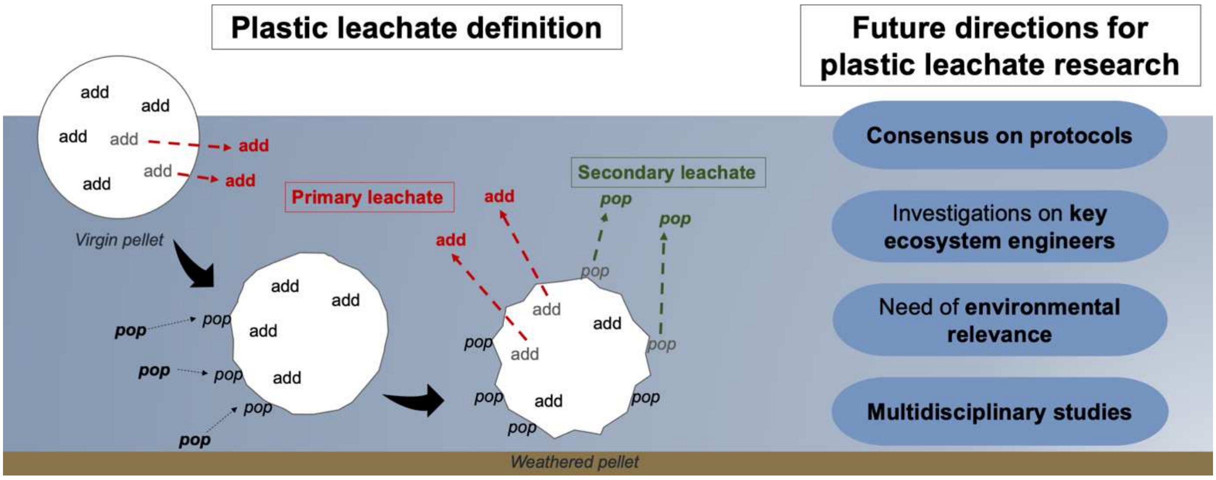
Graphical abstract



Abstract

With 4 to 12 million tons of plastic entering the marine environment each year, plastic pollution has become one of the most ubiquitous sources of pollution of the Anthropocene threatening the marine environment. Beyond the conspicuous physical damages, plastics may release a cocktail of harmful chemicals, i.e. monomers, additives and persistent organic pollutants. Although known to be highly toxic, plastic leachates seemingly appear, however, as the “somewhat sickly child” of the plastic pollution literature. We reviewed the only 26 studies investigating the impact of plastic leachates on marine microbes and invertebrates, and concluded that the observed effects essentially depend on the species, polymer type, plastic composition, accumulated contaminants and weathering processes. We identified several gaps that we believe may hamper progress in this emerging area of research and discussed how they could be bridged to further our understanding of the effects of the compounds released by plastic items on marine organisms. We first stress the lack of a consensus on the use of the term ‘*leachate*’, and subsequently introduce the concepts of primary and secondary leachates, based on the intrinisic or extrinsic origin of the products released in bulk seawater. We discuss how methodological inconsistencies and the discrepancy between the polymers used in experiments and their abundance in the environment respectively limit comparison between studies and a comprehensive assessment of the effects leachate may actually have in the ocean. We also discuss how the imbalanced in the variety of both organisms and polymers considered, the mostly unrealistic concentrations used in laboratory experiments, and the lack of investigation on key ecosystem engineers may considerably narrow the spectrum of our understanding of the plastic leachates’ effects. We finally discuss how increasing multi-disciplinarity through collaborations between different research fields may benefit to an area of research which is still in its early infancy.

Keywords: plastic leachate, plastic pollution, marine invertebrates, marine microbes, marine algae

1. Introduction

Following the early visionary description of the potentially infinite applications of plastics and the predicted advent of the so-called ‘plastic era’ (Yarsley and Couzens, 1942), yearly plastic pollution skyrocketed from 1.5 million tons in 1950 to 368 million tons in 2019 (PlasticsEurope, 2020) and the staggering 9 billion tons (9×10^9 t) produced so far are expected to increase sixfold by the mid-21st century (Zalasiewicz et al., 2016). Plastics are also increasingly discarded (6.3×10^9 tons of total plastic waste in 2015; Geyer et al., 2017), resulting in alarming accumulation of debris in landfills or environments (ca. 5×10^9 tons between 2015 and 2017; Geyer et al., 2017). As low-density, decay-proof and commonly robust materials, easily transported by wind and water, plastics have become one of the most ubiquitous and conspicuous sources of pollution of the Anthropocene, threatening the environment, the economy and human well-being on a global scale (Amelia et al., 2021; Galloway, 2015; Nelms et al., 2017), and the potential threats presented by plastic debris have been identified as a major global conservation issue and a key priority for research (GESAMP, 2010; Harrison and Hester, 2019; Vegter et al., 2014).

Beyond the growing awareness of anthropogenic litter as a critical environmental problem, plastics are now widely reported within the marine environment (Browne et al., 2010; De-la-Torre et al., 2020; Nelms et al., 2017). Noticeably, plastics represent 50 to 90% of the total litter found on shorelines and seafloors (Agamuthu et al., 2019), and up to 100% of floating debris (Galgani et al., 2015). The situation may even get worse as every year 4 to 12 million tons of plastics enter the marine environment, a figure expected to increase by an order of magnitude by 2050 (Jambeck et al., 2015). The majority of these plastic litter originates from land via inland waterways and wastewater outflows, but fisheries and aquaculture are also generators of plastic wastes. Once released into the marine environment plastic debris have the potential to disperse via wind and currents (Jambeck et al., 2015) and accumulate in coastal environments (Lebreton et al., 2019).

Macroplastics (i.e. plastic items larger then 5mm) are perceived as one of the most concerning form of plastic pollution due to their ubiquitous presence in the environment, such as the deposition of beached debris (Barnes et al., 2009). Macroplastics cause conspicuous physical damages, as the injuries causes by ingestion of plastic debris (Andrady, 2011) or the entanglement of marine fauna (Andrady, 2011; Gall and Thompson, 2015) through lost fishing nets or traps (Browne et al., 2015). These debris can also lead to the alteration, and even the destruction, of habitats, e.g. lost lobster traps on seabed cause a 14 – 20% reduction in the corals' cover (Uhrin et al., 2014).

Plastic pollution is, however, also related to microplastics (i.e. plastic smaller than 5 mm; Law and Thompson, 2014) and nanoplastics (i.e. smaller than 1 µm; Cole and Galloway, 2015). Macroplastics are a major contributor to microplastics due to their fragmentation through natural weathering process, i.e. mechanical abrasion and photochemical oxidation (Andrady, 2011), hence called secondary microplastics. On the other hand, primary microplastics may also directly enter the marine environment, especially originating from plastic industry, i.e. plastic pellets, microfibers from clothing or microbeads used in cosmetics (Hermabessiere et al., 2017). Altogether, microplastics are now considered as the most numerically abundant form of solid waste on Earth (Eriksen et al., 2014) and represent a potential threat to marine ecosystems globally (Galloway et al., 2017). Noticeably, they constitute a far more pernicious source of pollution than macroplastics with a large range of detrimental effects on marine life. Their ingestion prompts the desorption of the chemical pollutants adsorbed onto their surface which causes adverse effects (Avio et al., 2015). Furthermore, the subsequent accumulation into tissues and organs leads to cascade through the food chain (Desforges et al., 2015); see also Galloway et al. (2017) and de Sá et al. (2018) for recent reviews.

In addition to physical damages, it is noticeable that plastic polymers can be intrinsically harmful. For instance, a study assessing the toxicity of 55 plastic polymers based on their chemical composition (Lithner et al., 2011) found that 31 of them are made of monomers that belong to the two worst of the ranking model's five hazard levels, i.e. levels IV–V that are classified either as carcinogenic, mutagenic or toxic for reproduction. Beyond the toxicity of the polymers themselves, two main categories of harmful chemicals may typically originate from plastics: (i) the ever growing number of additives (i.e. over 400 so far as listed under the mapping exercise of the European Chemical Agency plastic additives initiative; www.echa.europa.eu) that enter the composition of plastics when manufactured such as light and heat stabilizers, antioxidants, nucleating and antistatic agents, flame retardants, plasticizers and colorants (Hahladakis et al., 2018; Hermabessiere et al., 2017), and (ii) the wide range of anthropogenic contaminants that accumulate onto plastic surface (Fries and Zarfl, 2012) at concentrations reaching up to 6 orders of magnitude higher than those found in the surrounding water (Hermabessiere et al., 2017). These latter chemicals include a variety of persistent organic pollutants (POP) such as polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), polybrominated diphenyl ethers (PBDE), pesticides or dichlorodiphenyl-trichloroethane (DDT) (Kedzierski et al., 2018; Ogata et al., 2009).

Both the accumulation of these chemicals onto, and their release from, plastic surface are a function of salinity, UV exposure, and environments enriched in organic matter (Delle Site, 2001). In addition, studies conducted on both non-weathered and weathered plastic products of various polymer types (e.g. Bejgarn et al., 2015; Gandara e Silva et al., 2016; Li et al., 2016; Lithner et al., 2009, 2012; Seuront, 2018; Seuront et al., 2021), suggest that plastics release significant amounts of hazardous chemicals over their lifetime. As an example, a recent study (Gardon et al., 2020) identified 7 additives—including 6 phthalates (DMP, DEP, DBP, BBP, DEHA and DEHP) and an antioxidant (Irgafos 168®)—and 20 PAHs released by new and aged plastic gear used in the pearl-farming industry. Specifically, though both additives and POPs are known to be either toxic, carcinogenic, mutagenic or endocrine disruptors (Weis, 2019), the sub-lethal effects of these hazardous chemicals have still, however, been relatively overlooked in the plastic pollution literature, despite the very active nature of this research field.

To illustrate this aspect further, we used the Web of Science (accessed February 7, 2022) which returned 42,506 papers included the words '*plastic*' and '*pollution*' (i.e. '*plastic*' and 'pollut*') in their topic since 1950 (i.e. the beginning of industrialization of plastic production), and this figure has since been growing exponentially (Fig. 1a). Noticeably, however, only 526 papers include the term '*leachate*' (i.e. '*plastic*' and 'pollut*' and 'leachate*') in their topic (Fig. 1b), a word often used in a generic way to refer to the release of hazardous chemicals from plastics into the environment in the absence of ingestion. More specifically, when considering the research areas '*Environmental Sciences and Ecology*' and '*Freshwater and Marine Ecology*', figures drastically dropped by ca. 99% when looking for leachate (i.e., 35,646 to 501 and 7,064 to 107 papers, respectively for each field; Web of Science, accessed February 7, 2022; Fig. 1). Even more noteworthy is the number of papers assessing the impact of plastic leachates i.e., free from particles, on marine microbes and invertebrates that only reaches 26 papers (see Supplementary Materials Table S1). Considering that plastic leachates are likely to be ubiquitous in marine environments as they are likely to be produced over the plastic lifetime, especially during the continuous process of fragmentation, we stress that addressing the origin, nature and impact of plastic leachates is of upmost importance for the future of plastic pollution research.

In this context, the present work aims at critically reviewing the blossoming research area devoted to plastic leachates. Specifically, we first identify and discuss the terminological ambiguity in the use of the word '*leachate*' in the literature, and subsequently suggest a tentatively universal definition of leachates in the context of plastic pollution. Next, we critically review the literature assessing the biological effects of leachates on marine microbes and invertebrates through the identification of clear discrepancies between the organisms and polymers considered in the literature and plea for more unified approaches. Finally, we discuss future directions that the research on plastic leachates impacts on marine life may need to follow to reach a better understanding of the potential effects of the hidden side of a pernicious component of the ever-increasing plastic pollution.

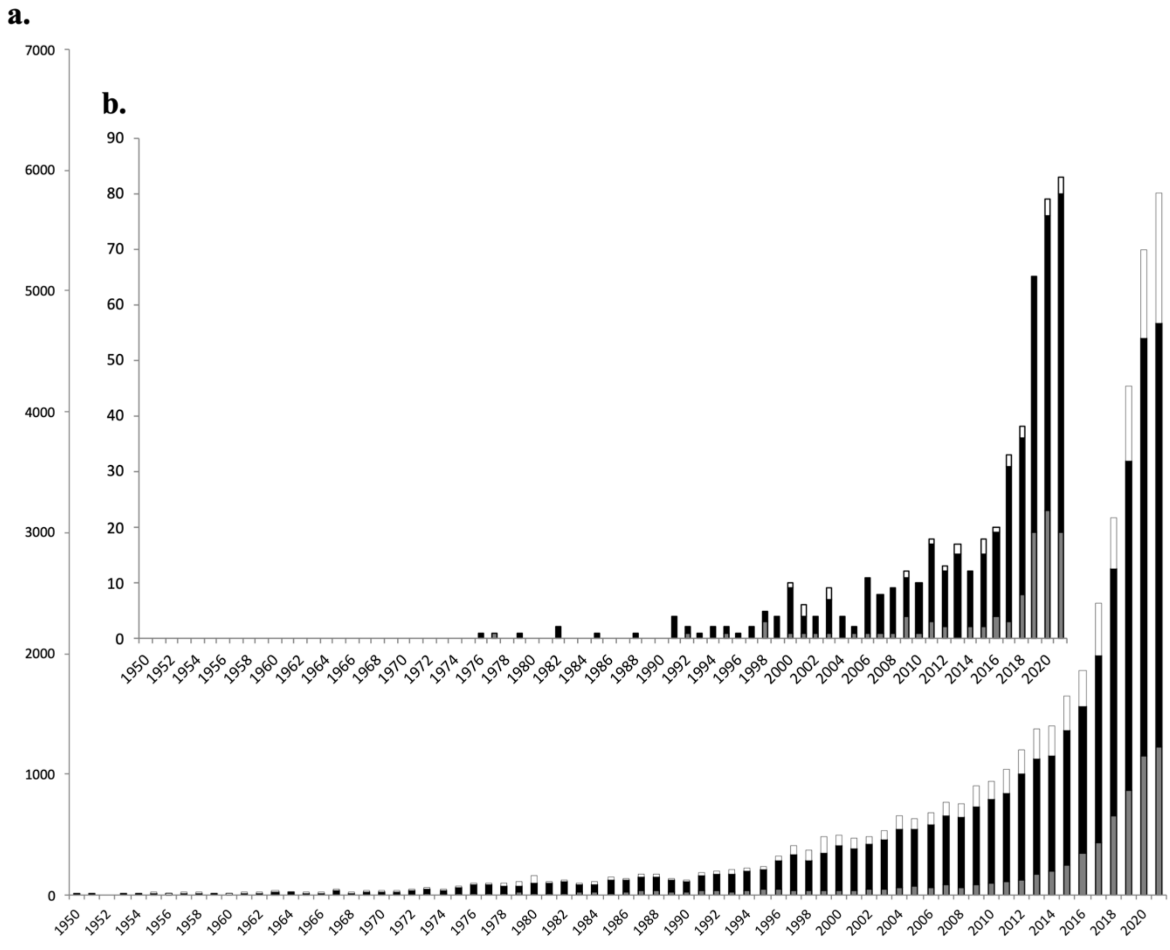


Figure 1. Temporal evolution of the number of papers published between 1950 (i.e. beginning of the industrialization of plastic production) and 2021 that include (a) plastic and pollution (i.e. *plastic* and pollut*) or (b) plastic and pollution and leachate (i.e. *plastic* and pollut* and leachate*). The total bar represents the number of papers in the global field. The part of papers in Environmental Sciences and Ecology is in black among which the part of papers in Marine and Freshwater Biology is in grey.

2. What does '*leachate*' actually mean: towards a process-based terminological consensus

In the area of research devoted to marine biology, the impact of plastic leachates on fauna was first investigated in the early nineties (Weis et al., 1992). Although plastic accumulation in the oceans is now conspicuous and ubiquitous, and widely debated in areas ranging from molecular biology to sociology and politics, the number of publications related to plastic leachates remained rather anecdotal until 2021, as more than 65% of the plastic leachate literature (i.e., 17 out of 26 papers) have been published since 2020. Even more noticeably and unexpectedly, a clear and non-ambiguous definition of plastic leachate is still critically lacking. To the best of our knowledge, only three papers actually explicitly defined the word '*leachate*', i.e. "*the capacity of [chemical compounds] to be desorbed (leached)*" (Nobre et al., 2015), the "*desorption of chemicals into the surrounding environment in the absence of plastic ingestion*" (Gardon et al., 2020) and "*the ability of plastic to transfer POPs to biota via desorption and subsequent dermal absorption (i.e. leaching)*" (Coffin et al., 2018). These definitions noticeably appear to be context-dependent, which worsen the actual lack of consensus. Fundamentally, the subsequent terminological ambiguities may potentially ultimately be detrimental to the development of this research field, by analogy to what has been shown for scientific progress in general (Popper, 2002).

Noticeably, the 26 papers that assessed the impact of plastic leachates on marine microbes and invertebrates indistinctly used the words '*leachate*', '*release*' and '*desorption*' to refer to the transfer of chemicals from plastic to the surrounding seawater and sediment. *Sensu stricto*, the term '*release*' is by far the most generic in the sense that is does not refer to any chemical nor physical process, but describes "*a substance that is allowed to flow out from something*" (Cambridge Dictionary). In contrast, '*desorption*' and '*leaching*' have much more specific meanings. Desorption is the process by which molecules (essentially POPs) adsorbed onto a surface or molecules (specifically here additives) or absorbed (i.e. incorporated) into a solid are released (Schaschke, 2014). In turn, leaching is a separation process by which a soluble component is removed from a solid by the action of a percolating solvent (Backhurst, 2002) i.e., a fluid passing through the substance. As a consequence, the term '*leachate*' does not cover all the chemical and physical processes leading to the transfer of contaminant from plastics to the environment.

In this context, the definition of leachate provided by Gardon et al. (2020) i.e., "*desorption of chemicals into the surrounding environment in the absence of plastic ingestion*", is relevant given that they investigated the release of both additives and POPs that are respectively *absorbed* i.e., intrinsically bounded to the polymer during the manufacturing process, and *adsorbed* to plastic polymers. To overcome the intrinsic limitation related to the term '*desorption*' which indistinctively refers to the desorption of absorbed and adsorbed molecules, and given the fact that the word '*leachate*' has now been extensively used and acknowledged in the plastic literature, we suggest to refine the use of the term '*leachate*' as primary and secondary leachates to describe the release of molecules that are respectively absorbed and adsorbed to plastic polymers (Fig. 2).

This terminological distinction is particularly relevant in the context of the research conducted on the effects of the molecules released by non-weathered plastics and weathered plastics (e.g. Cormier et al. 2021; Gardon et al. 2020; Gewert et al. 2021; Koski et al. 2021; Lithner et al. 2009, 2012; Nobre et al. 2015; Sarker et al. 2020; Seuront 2018). Non-weathered (i.e. virgin) plastics include plastic consumer products (e.g. DVD-case, bags, food packaging, cups and bottles) and plastic pellets (i.e. raw resin pellets used in the manufacture of plastic products; Hidalgo-Ruz et al., 2012) that have not been exposed to any weathering process such as biodegradation, heat and UV irradiations, physical abrasion or oxidation (Liu et al., 2020b). Once discarded in the ocean, these items are a source of primary leachates (Fig. 2). In contrast, weathered plastics include both naturally and experimentally weathered plastic items. Naturally weathered plastics are discarded plastic consumer products of various origins that are found stranded on beaches, floating at the surface of the ocean or sank on the ocean seafloor; they range from macroplastic items such as tires, fishing nets and down to primary microplastics such as pellets accidentally lost during the process of production, transport and manufacturing and secondary microplastics resulting from the continuous fragmentation of larger plastic items. These items, during their stay in the ocean accumulate (i.e. adsorb), either through hydrophobic and electrostatic interactions, and non-covalent bounding such as van der Walls forces (see Fu et al. (2021) and Joo et al. (2021) for reviews), anthropogenic pollutants on their surface, e.g. heavy metals, PCBs, PAHs, DDT, PBDE (Kedzierski et al., 2018; Ogata et al., 2009), that are potentially released as secondary leachates (Fig. 2). Note, however, that these plastic items are also intrinsically a source of primary leachate (Fig. 2).

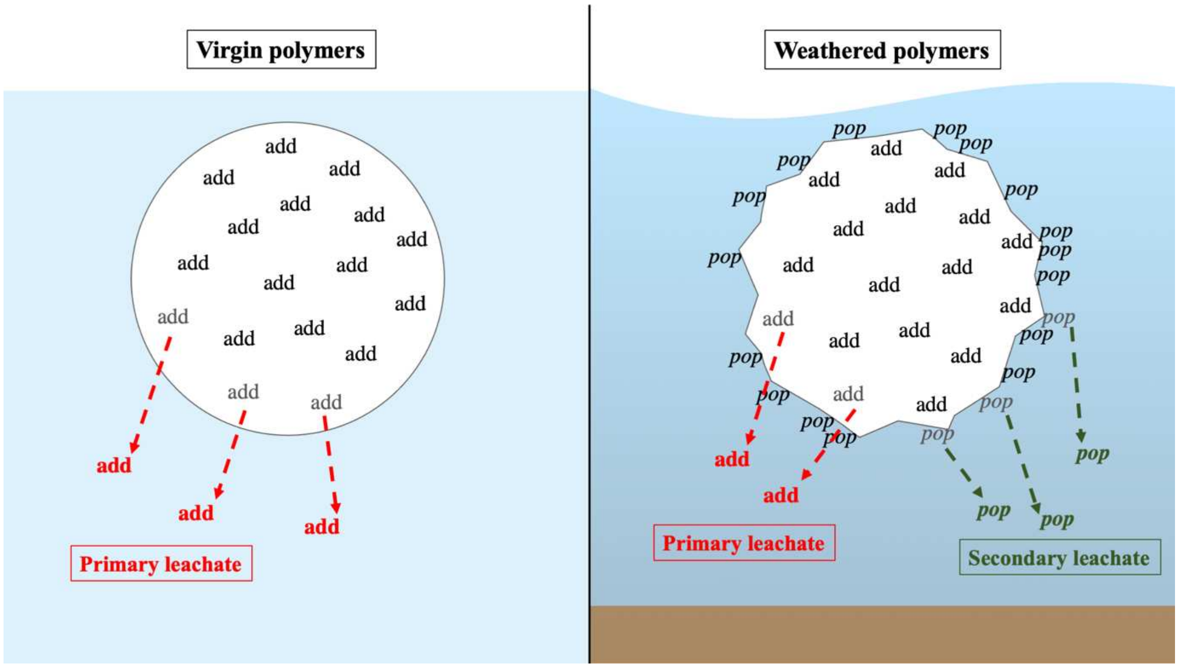


Figure 2. Composition of plastic leachate of virgin and aged, i.e. that have stayed in the environment, polymers. add: plastic additives, pop: persistent organic pollutants.

The release of additives from plastic particles is facilitated by both their lack of covalent bound to the plastic molecules and their low molecular weight. The amount of released additives further depends on a range of factors including (i) the proportion of additives used in the manufacture of the polymer (e.g. PVC typically contain more than 40 % by weight of plasticizers, mostly phthalates; Andrade et al., 2021), (ii) the permeability of the polymer structure, which is driven by its physical state (e.g. the size of the gaps in rubbery polymers are much larger than in glassy and crystalline ones, hence rubbery polymers are characterized by higher release rates), and (iii) the molecular weight, shape and polarity of additives, i.e. lighter and linear additive molecules will be release at faster rates than heavier and branched ones (Brydson, 1999; Gilbert, 2017). Alternatively, virgin plastics can be experimentally aged (Bejgarn et al., 2015; Martínez-Gómez et al., 2017), which prevent the accumulation of environmental pollutants in order to understand the impact of weathering process on the desorption of additives.

3. Methodological issues

3.1. Keeping (or not?) plastic particles in the leachate solution to assess their effect?

The effects of leachates on marine organisms have typically been evaluated from both virgin and weathered plastics by putting a given number of plastic items in seawater where they were left for a given incubation time. The subsequent biological assays were conducted on leachate solutions separated from plastic items by filtration (e.g. Gardon et al., 2020; Ke et al., 2019; Piccardo et al., 2020; Tetu et al., 2019), but also on leachate solutions where plastic items were then kept in suspension (e.g. Avio et al., 2015; Gandara e Silva et al., 2016; Silva et al., 2020; Song et al., 2020; Tang et al., 2018). Though the former is appropriate to assess the effects of primary and secondary leachates on marine organisms, the latter may be misleading as it might be challenging to decipher the physical effects due to the presence (and eventually the ingestion) of plastic particles from the chemical effects that can be exclusively related to the leachate. To the best of our knowledge, only 26 papers investigated the impact of leachate *sensu* *stricto*, i.e. without particles (Aminot et al., 2020; Bejgarn et al., 2015; Capolupo et al., 2021, 2020; Chae et al., 2020; Cormier et al., 2021; Gardon et al., 2020; Gewert et al., 2021; Ke et al., 2019; Koski et al., 2021; Langlet et al., 2020; Lehtiniemi et al., 2021; Li et al., 2016; Martínez-Gómez et al., 2017; Nobre et al., 2015; Oliviero et al., 2019; Piccardo et al., 2020; Rendell-Bhatti et al., 2021; Sarker et al., 2020; Schiavo et al., 2021; Seuront, 2018; Seuront et al., 2021; Tetu et al., 2019; Thomas et al., 2020; Trestrail et al., 2020; Weis et al., 1992). Among these papers, 4 investigated the physical impacts alongside the chemical impacts, by exposing their organisms to both leachate *per se* and solution still containing particles (Martínez-Gómez et al., 2017; Nobre et al., 2015; Oliviero et al., 2019; Piccardo et al., 2020). Noticeably, the difficulty in deciphering the physical and chemical effects of plastics in the leachate-oriented literature also exists in the literature investigating the physical effects of plastic particles on marine organisms. For instance, to the best of our knowledge, among the 8 papers (Bhattacharya et al., 2010; Davarpanah and Guilhermino, 2015; Long et al., 2015; Prata et al., 2018; Sjollema et al., 2016; Su et al., 2020; Zhang et al., 2017; Zhu et al., 2019) assessing the impact of microplastic particles on marine phytoplankton, only 2 papers mentioned that toxic substances may be released by microplastics in their discussion (Su et al., 2020; Zhang et al., 2017).

In this context, the next section specifically focuses on papers investigating the impact of plastic-free leachate solutions on microbes and invertebrates.

3.2. Imbalance in organisms and polymers considered

To examine in further details the research effort devoted to assess the impact of plastic leachates on marine organisms, from the 26 papers listed above, we estimated the number of organisms and polymers assessed, identified the overall number of experiment conducted (e.g. a paper investigating the impact of the leachate from 3 type of PVC particles from different origins on a single species, i.e. blue PVC, green PVC and orange PVC originating from different toys on *Paracentrotus lividus* (Oliviero et al., 2019), would be considered as 3 separate experiments), and subsequently estimated the research effort devoted to each a species, a phylum or a polymer type. The plastic particles without any chemical indication or only partial indications on the polymer type were considered as unknown polymers, i.e. beached plastics, plastic products without manufacturer indication, particles for which polymer is supposed because of its color or texture but without chemical proof.

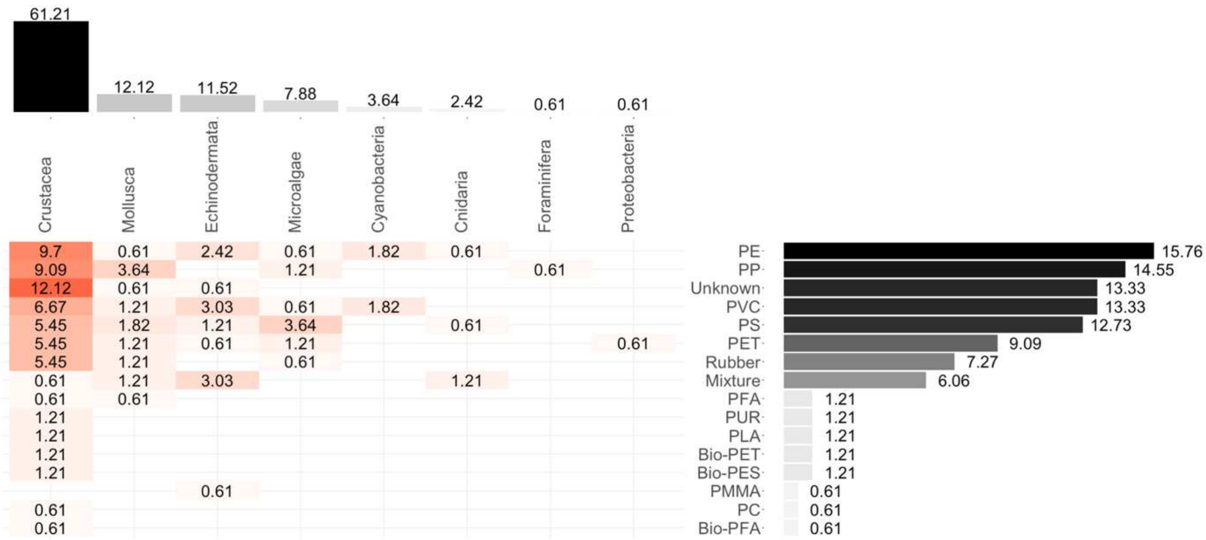


Figure 3. Heatmap analysis of the research effort in the area of research devoted to the impact of plastic leachates on coastal invertebrates, microorganisms and plants. The more intense the red is, the more experiments have been made on the polymer-phylum couple considered. The percentage of research effort devoted to a polymer-phylum couple is written in the crossing box. Barplots represent the percentage of research effort devoted to a specific polymer or phylum.

Eighty-seven percent (87%) of the research effort is focused on pluricellular organisms (i.e. crustaceans, mollusks, echinoderms and cnidarians), versus 13% on unicellular organisms (i.e. foraminifera, microalgae and bacteria; Fig. 3). One of the most striking pieces of evidence coming out of our literature review, is that crustaceans appear to be a highly considered taxon, as it accounts for ca. 61% of the research effort (Fig. 3), especially *Nitocra spinipes* which accounts for more than 50% of the research effort including the vast majority of the less-tested polymers (i.e. less than 1.3% of the research effort, e.g. bio-polymers, PLA, PUR). Mollusks, echinoderms and microalgae account for respectively ca. 12, 12 and 8%, whereas cyanobacteria, cnidarians, foraminifera and proteaobacteria represent less then 4% of the research effort (Fig. 3). In addition, the impact of plastic leachates on foraminifera and proteobacteria was investigated for only one type of polymer, i.e. PP and PET respectively (Fig. 3). PE, PP, PVC, PS and PET are the most studied and account respectively for ca. 16 to 9% of the research effort (Fig. 3). The impact of rubber and mixtures of polymers leachates are respectively ca. 7 and 6% of the research effort (Fig. 3). In contrast, the remaining studied polymers account for less than 1.3% each (Fig. 3). It is also noticeable that the over-representation of crustaceans also manifests itself in terms of the polymer-species combination found in the literature. The first 7 polymer-species couples considered, are crustaceans exposed to leachates, respectively, from unknown polymer (12% of the research effort), PE (ca. 10%), PP (ca. 9%), PVC (ca. 7%), PS (ca. 6%), PET (ca. 6%) and rubber (ca. 6%; Fig. 3). The remaining polymer-species couples account for less than 4% of the research effort.

4. Effect of plastic leachate on marine microbes and invertebrates: state-of-the-art.

The experimental conditions, i.e. polymer concentration, leaching time, exposure time, observed biological parameters and observed effects, of each experiment investigating the impacts of plastic leachates on coastal microbes and invertebrates are gathered in Supplementary Materials 1 Table S1.

4.1. Unicellular organisms

Though unicellular organisms still have barely been considered in a plastic leachate context (Fig. 1), contradictory results were found in the very few organisms exposed to plastic leachates. Specifically, bacteria and microalgae were impacted at various levels (see below), in sharp contrast to benthic foraminifera which were not affected either behaviorally nor physiologically (Langlet et al., 2020). Noticeably, the exposure of the proteobacteria *Vibrio fischeri* to PET leachates induced an increase in its natural bioluminescence which was hypothesized as a potential early sign of chronic stress (Piccardo et al., 2020). In addition, the cyanobacteria *Plochlorococcus* sp. showed a decrease in photosynthetic activity, membrane integrity and population growth when exposed to virgin (Sarker et al., 2020; Tetu et al., 2019) and aged (Sarker et al., 2020) PE and PVC leachates. Similarly in microalgae, the exposition to PS leachates induced an increase in chlorophytes photosynthesis activity (*Dunaliella salina*, *Scenedesmus rubescens*, *Chlorella saccharophila* and *Stichococcus bacillaris*; Chae et al., 2020) and PS, PE, and PP leachates inhibited growth and increased ROS production and DNA damages in *Dunaliella tertiolecta* (Schiavo et al., 2021). In addition, diatoms population growth changes depend on polymer types; population growth decreased when *Skeletonema costatum* was exposed to PP, PS, PVC and CTR leachates, whereas PET leachate did not affect *S. costatum* (Capolupo et al., 2020) and induced an increase in the population growth *Phaeodactylum tricornutum* exposed to lower concentration (Piccardo et al., 2020). Though still in its early age, the research devoted to assess the impact of plastic leachate on unicellular organisms nevertheless suggests that leachate toxicity may be phylum-dependent.

4.2. Pluricellular organisms: leachate effects at different levels of biological organization

4.2.1. Oxidative stress

The research related to the assessment of the molecular impacts of plastic leachate is based on modifications in biomarkers, i.e. the first signals of biological effects that are induced by toxicants (Capolupo et al., 2021). To the best of our knowledge, these biomarkers were only investigated in the mussel *Mytilus galloprovincialis* and oxidative stress was reported, inducing detoxification processes depending on the polymer type. The strongest effect was observed after an exposition to leachate from PS, then PET and finally PS, PVC and CTR (Capolupo et al., 2021). In contrast, PFA leachate did not induce any damage (Trestrail et al., 2020), despite the use of experimental concentrations more than one order of magnitude higher than those considered for the other polymers. Additionally, whereas PVC leachate was shown to be neurotoxic (Capolupo et al., 2021), this was not the case for other polymers such as PET, PS, PP, CTR (Capolupo et al., 2021) and PFA (Trestrail et al., 2020). Finally, metal (used as additives in some polymers to confer specific properties) exposure was investigated and the exposition to PET, PS, PVC, PP and CTR leachates did not reveal any change in *M. galloprovincialis* (Capolupo et al., 2021). These results suggest that toxicity of plastic leachates is likely to depend on polymer type.

4.2.2. Cellular integrity and embryonic development

The impacts of leachates on the embryonic development and the cellular integrity have been investigated only in echinoderms and mollusks. Specifically, PE leachate induces abnormal embryonic developments in the sea urchins *Lytechinus variegatus* (Nobre et al., 2015) and *Paracentrotus lividus* (Cormier et al., 2021; Martínez-Gómez et al., 2017). These results contrast with the effects of leachates from virgin PE free from additives on *P. lividus* (Rendell-Bhatti et al., 2021). Similarly, the embryonic development of *P. lividus* was impaired by an exposition to leachates from PET (Piccardo et al., 2020), both colored (Oliviero et al., 2019) and common white PVC (Rendell-Bhatti et al., 2021), PMMA (Thomas et al., 2020) and beached pellets mainly composed of PE (Rendell-Bhatti et al., 2021) and of a mixture of PE and PP (Cormier et al., 2021). In turn, beached pellet leachates have no effect on the development of *L. variegatus* embryos (Nobre et al., 2015). As the tested concentration was similar to the previously mentioned PE leachate (Nobre et al., 2015), these results suggest that leachate toxicity is likely to depend on the cocktail of additives and pollutants that are released during the leaching process. No conclusions can be drawn on the impact of PS leachate on the embryonic development of *P. lividus* as contradictory outcomes have been reported, i.e. significant decrease (Martínez-Gómez et al., 2017) *vs.* no significant changes (Thomas et al., 2020). In addition, the leachate solution's concentrations are reported in different units (i.e. number of microspheres per liter vs. mg mL^{-1}; Supplementary Materials 1 Table S1), preventing any comparisons. Furthermore, development abnormalities were observed in the mussels *Meretrix meretrix* exposed to PE leachate (Ke et al., 2019) and *Mytilus galloprovincialis* exposed to PET, PS, PVC, PP and CTR leachates (Capolupo et al., 2020), and in the oyster *Pinctada margaritifera* exposed to the leachate of virgin and weathered plastic gears made of a mixture of PE and PP originated from pearl-farms (Gardon et al., 2020). The cellular integrity of bivalve is also affected by plastic leachate, with PP, PVC and CTR leachates were the most damaging on *M. galloprovincialis*, followed by PS leachate and PET leachate (Capolupo et al., 2021, 2020).

4.2.3. Mortality

Mortality is by far the most studied biological parameter in the investigation of plastic leachates' impacts, both on larvae and adults. Mortality increased for each polymer type tested in barnacle larvae (*Amphibalanus amphitrite* exposed to PC, HDPE, LDPE, PET, PP, PS and PVC leachates; Li et al., 2016) and crustacean larvae (*Artemia* sp. exposed to PFA and bio-PFA leachates; Trestrail et al., 2020). Noticeably, jellyfish larvae were, however, not sensitive (*Aurelia* sp. exposed to PE and beached pellets leachates; Cormier et al., 2021). Nonetheless, *Aurelia* sp. larvae were exposed to lower concentration of leachates than *Artemia* sp. larvae, and the concentration tested on *A. amphitrite* larvae was in a different unit; conclusions cannot thus be drawn easily.

The mortality of adult mussels exposed to PP, PVC, CTR (*Mytilus galloprovincialis*; Capolupo et al., 2020), virgin and aged mixture of PE+PP (*Pinctada margaritifera*; Gardon et al., 2020) and PE (*Meretrix meretrix*; Ke et al., 2019) leachates increased, whereas it did not with PET and PS leachates (*M. galloprovincialis*; Capolupo et al., 2020). The mortality of the adult copepod *Nitocra spinipes* also increased following an exposure to the leachates of PUR, aged PIR and bio-PES particles (Bejgarn et al., 2015). In contrast, no significant change was observed with PE, PET, PP (Bejgarn et al., 2015; Gewert et al., 2021), PLA and bio-PET leachates (Bejgarn et al., 2015). Similarly, the mortality of the adult copepod *Limnocalanus macrurus* was not impacted by the exposition to LDPE (from virgin granules and vegetable package; and SBR leachates (Lehtiniemi et al., 2021). Taken together, these results suggest that the survival of adult copepods to plastic leachate typically depends on the polymer type and the species; see Bejgarn et al. (2015), Capolupo et al. (2020), Gewert et al. (2021) and Lehtiniemi et al. (2021).

Noticeably, the toxicity of plastic leachates may also depend on weathering processes, e.g. mechanical abrasion and photochemical oxidation (Andrady, 2011). For instance, artificial UV exposure triggered a toxicity in every polymer tested (i.e. PE, PET, PP, PS pellet, PS, PVC) on the *Nitocra spinipes* adult survival, although the great majority (i.e. PE, PET, PP, PS pellet) were not toxic before the weathering process (Gewert et al., 2021). The impact of the weathering process on toxicity is dependent on the polymer origin, i.e. composition, as PP leachate became toxic after irradiation for *N. spinipes* whereas PUR leachate lost its toxicity (Bejgarn et al., 2015).

Moreover, additives composition is likely to have a great role in toxicity of leachates as the *Nitocra spinipes* adult survival was different depending on the origin of plastic particles. Among PVC leachates originated from 3 different objects, 2 leachates induced an increase in *N. spinipes* mortality whereas the last one did not (Bejgarn et al., 2015). Similarly, leachates from PS food packaging induced mortality whereas leachate from PS pellet did not (Gewert et al., 2021). Even weathering processes have different impact on toxicity depending on plastic origin, i.e. PVC packaging became toxic after irradiance whereas PVC from garden hose lost its toxicity (Bejgarn et al., 2015). Consequently, for a same polymer, toxicity can be different, which emphasize the importance of additives in the toxicity of plastic leachates and on the impact of weathering processes.

4.2.4. Behavior and cognition

Organisms' behaviors have been investigated in mussels, gastropods, in which significant changes have been described, and copepods. Specifically, the aggregation behavior of different mussel species was studied. The percentage of aggregated mussels, the time to aggregate and the crawling distance increased, whereas the byssal thread production was not impacted by the exposition to PP leachate, for *Choromytilus meridionalis* and *Mytilus edulis* (Seuront et al., 2021). The opposite was observed for *Mytilus galloprovincialis* and *Perna perna* (Seuront et al., 2021). Consequently, the strategy adopted when mussels are exposed to plastic leachate seems to be species-dependent, and driven by their intrinsic dominant trait (Seuront et al., 2021). In the gastropod *Littorina littorea* a decrease in vigilance and antipredator behaviors was shown when exposed to PP and beached pellets showed (the beached pellet being the more toxic; Seuront, 2018), which might have an impact on its survival. In contrast, the swimming behavior of the copepod *Limnocalanus macrurus* was not impaired by an exposition to any plastic leachate (i.e. LDPE from virgin granules, vegetable package, and SBR from artificial turf or recycling factory; Lehtiniemi et al., 2021).

4.2.5. Reproduction

The impact of plastic leachates on the reproduction has been studied on crustaceans (copepods and barnacles), mollusks (mussels), and echinoderms (urchins), through the measurement of egg production, fertilization, hatching or settlement. The nauplii's settlement of the barnacle *Amphibalanus amphitrite* decreased irrespective of the polymer type (Li et al., 2016). Noticeably, the impacts of rubber leachate on copepods depend on its origin. The exposition to virgin and aged CTR leachates had no effect on the egg production and hatching of the copepods *Acartia tonsa* and *Temora longicornis*, whereas an exposure to TWP leachate caused an increase in *A. tonsa* egg production but not on hatching (Koski et al., 2021). In addition, no changes in fertilization were observed neither in the sea urchin *Paracentrotus lividus* exposed to PMMA and PS leachate (Thomas et al., 2020), nor in the mussel *Meretrix meretrix* exposed to PE leachate (Ke et al., 2019). On the contrary, the exposition to PET, PS, PVC, PP and CTR leachate induced a decrease in the gamete fertilization of the mussel *Mytilus galloprovincialis* (Capolupo et al., 2020). Consequently, results revealed that the impacts of plastic leachates on reproduction may depends on polymer type and species. Note however that leachate concentrations and units are not homogenous between studies preventing any inter-study comparisons.

5. The future of plastic leachate research: perspectives toward an environmental and unified approach

In the present work we showed that the area of research devoted to plastic leachates is still at its very early beginning and, as detailed above, is suffering from both relatively serious conceptual and methodological gaps, and a critical lack of consensus on what the term 'leachate' actually means. These gaps are likely to hamper our ability to understand the actual ecological effect of this new and pernicious type of pollution. In this context, we claim that plastic leachates-related research would largely benefit from the adoption of conceptual, terminological and methodological consensuses within the scientific community. As for now, comparisons between studies are hardly possible since the units used are often different; see Supplementary Materials 1 Table S1). Similarly, the range of both concentrations (i.e. from 0.0003 to 100 mg/mL) and size (i.e. 1 µm to 10 cm^3) of plastic particles used to prepare leachate solutions as well as leaching times (i.e. 24 hours to 28 days) and exposure times (i.e. 15 min to 7 days) used in the leachate-related biological assays consistently vary dramatically between studies (Supplementary Materials 1 Table S1), which then become barely comparable. We also identified a remarkable disequilibrium in both organisms and polymers that have been considered in studies so far (Fig. 3).

5.1. On the need to use realistic concentration of plastic particles

Typical environmental plastic concentrations range between ng to µg/L (Lenz et al. 2016) or 1-500 particles/m^3 (Koski et al., 2021), and can even reach up to ca. 17 mg/L in the intertidal zone (Paul-Pont et al., 2018). Although this issue has previously been pointed out (Lenz et al., 2016; Haegerbaeumer et al., 2019), experimental concentrations of plastic-related studies are still orders of magnitude higher, i.e. up to 100 g/L or 100,000 particles/L for plastic leachate studies (Supplementary Materials 1 Table S1), than what is usually found in the field. Though ecotoxicological investigations are relevant to identify general hazards and establish toxicity threshold for a given contaminant-organism pair (Paul-Pont et al., 2018), environmentally realistic concentrations should be systematically investigated in plastic leachate literature to understand the current impact and ecological consequences of plastic contamination on marine organisms.

5.2. Research effort should be fine tuned to the polymer type found in the environment

The polymers considered are not in complete accordance with what is found in natural environments. Noticeably, PE is the most abundant polymer type in natural aquatic environments (i.e. 23% of the abundance of polymers), followed by the group PP&A (i.e. PES, PLA and acrylics including PAM, PMMA and PVA; 20%), PP (i.e. 13%) and PS (i.e. 4%; Erni-Cassola et al., 2019). Specifically, in intertidal environments, PE and the group PP&A plastics are dominant (Pannetier et al., 2019), i.e. respectively 18% and 23% at a global scale, whereas PS, PP, PET and PVC particles only account for 6%, 5%, 3% and 3% respectively (Doyen et al., 2019; Erni-Cassola et al., 2019). Consequently, if PVC, PE, PET, PP and PS are over-investigated, polymers from the PP&A group (i.e. accounting for 3% of the research effort; Fig. 3) are greatly under-investigated and may probably deserve more attention. The disequilibrium between the polymers studied and their abundance in the environment may be explain by their toxicity or their high industrial demand. PUR, PAN and PVC are considered as the most hazardous polymer types according to their monomer composition, whereas PP, PLA, PET, PE and PS are evaluated to be among the least hazardous (Lithner et al., 2011). In this context, the over-investigation of PVC may be justified by its toxicity. In addition, the appeal for PET and PP may be explained by their high use in packaging industry (PlasticsEurope, 2020, 2018), and the ones for PE and PP by their general industrial demand (i.e. respectively 29,8 and 19,4 % of the 2019 plastics demand distribution by resin type; PlasticsEurope, 2020). Although investigating the hazardousness of each polymer type is important, it is now critical to explore the actual risks run by marine organisms in their natural habitat by focusing on polymers that are abundantly found in the environment.

5.3. Leachate solutions should be particle-free to avoid confounding effects

In section 3.1, we stressed that leachate solutions still containing plastic particles do not allow to decipher the relative contribution of the physical and chemical effects of plastics. It is hence of major importance to be specific about what type of leachate solution a study deals with, i.e. particle-free leachate solution *vs*. particle-loaded leachate solution, for the reader to be able to assess the relevance of the so-called leachate effects. As a consequence, it is critical to conduct experiments assessing the physical effects of plastics using additive-free polymers, and further run in-parallel experiments with particle-free leachate solution vs. particle-loaded leachate solution in order to unambiguously unravel the chemical and physical impacts of plastics.

5.4. From simple to chemically complex leachate soups

In the environment, organisms are exposed to a complex mixture of polymers which have undergone weathering processes constraining their toxicity and their size (Paul-Pont et al., 2018). Consequently, using only similar-sized virgin manufactured particles from a single polymer type is an unrealistic scenario considering the variety of plastic particles encountered in the environment. As previously stressed, plastic exposure involves organisms being potentially exposed to several sources of contaminants: (i) monomers, (ii) additives and (iii) POPs. Though known to be highly toxic (e.g. styrene monomers impaired the embryonic development of *Mytilus galloprovincialis*; Wathsala et al., 2018), the impact of monomers on marine organisms is barely investigated and should be considered in future investigations.

Furthermore, only 11/26 papers investigated the impact of naturally weathered plastics. Knowing that more than 5×10^9 tons of plastic are already accumulating in the environment (Geyer et al., 2017) against $4 - 12 \times 10^6$ tons entering marine environment each year (Jambeck et al., 2015), organisms are likely to be more exposed to secondary (i.e. sorbed contaminants) than primary leachates (i.e. manufactured additives). The former should hence deserve more attention. Consequently, in order to match experimental conditions with what is encountered by organisms in their environment, authors should rather mix beached and virgin particles, of different size, to assess a more realistic potential impact of plastic leachates on organisms. It would further allow to draw conclusions at an ecosystemic scale.

5.5. What organisms should be considered next?

As for polymer types, the diversity of studied organisms is not representative of the *in situ* diversity. As already stressed (Haegerbaeumer et al., 2019), meiofauna are still widely neglected and there is an urge to consider them in future investigations given their role in key processes such as bioturbation and related water-sediment fluxes. Noticeably, in contrast to crustaceans that are over-represented, some major ecosystem engineers are missing (Fig. 3, Supplementary Materials 1 Table S1). To the best of our knowledge, no studies have investigated the impact of plastic-free leachate on annelids or crabs (the impact of plastic-free leachate was investigated on the crab *Uca pugilator* to compare two materials and no negative control was performed; Weis et al., 1992), whereas they are considered as key organisms in bioturbation and bio-irrigation processes (Kristensen et al., 2012). Nonetheless, plastic-loaded leachate experiments revealed an impact of plastic exposition on the annelids *Hediste diversicolor* (i.e. PS nanoplastics impaired biomarkers expression and burrowing time; Silva et al., 2020), *Perinereis aibuhitensis* (i.e. PS microplastics increased mortality and reduced the rate of posterior segment regeneration; Leung and Chan, 2017) and *Arenicola marina* (i.e. polylactic acid, PE and PVC affected metabolic rates and sediment nutrient cycling; Green et al. 2016). These results suggest that plastic leachates would limit the contribution of these two ecosystem engineers to sediment reworking and bio-irrigation, which is likely to hamper the benthic ecosystem functioning. It further shows the importance of considering plastic leachates effects on species having different roles in the ecosystem functioning in order to understand their harmful effects at the ecosystem scale. Similarly, studies on macroalgae are missing, though they are structuring rocky-shore habitats. Adopting an ecosystemic point of view, hence considering the previously cited organisms in future investigations, is critical to conclude on the impact of plastic leachates on the ecosystem functioning.

5.6. Towards interdisciplinarity

Plastic has been a prolific research area (Fig. 1a) in numerous fields, from oceanography to ecology, by way of e.g., physics, economy and politics. Although complementary (e.g. chemists analyze the composition of plastic leachates, biologists explain their impacts on organism's biology and ecologists investigate the effects on the interactions between organisms and their environment), there is still a remarkable lack of collaboration between research fields. For instance, ecologists do not often analyze leachates composition (e.g. 13 % of the leachate experiments are driven with particles of unknown polymers; Fig. 3), though polymer composition varies greatly from one type to another, even for one manufacturer to another for a same polymer type. In addition, plastic particles following different path once discarded in the environment, they must accumulate, and then release, a unique mixture, leading to potentially very different effects. Hence, characterizing the type of polymers, and their composition in terms of primary and secondary leachates, to which a species would be exposed in its environment and/or experimentally appears of high importance to draw sound conclusions on the effect of plastic leachates.

Multidisciplinary research requires specific skills and tools (e.g. assessing the composition of plastic particles and leachates is particularly difficult as it is time-consuming, expensive and technical), and collaborations is the way forward. It is a matter of necessity to consider inter-disciplinarity for future investigations in order to further understand the source, the pathway and to predict the impact of plastic leachate at cellular, individual, community and ecosystemic scales.

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Supplementary Materials 1

Plastic leachates: bridging the gap between a conspicuous pollution and its pernicious effects on marine life

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Table S1. Effect of particle-free plastic leachate on marine microbes and invertebrates. Naturally weathered particles, e.g. beached pellets, are colored in light grey and artificially weathered particles are colored in dark grey. When known, the origin of the plastic is written under the polymer type in italic. Note points out that: chemical analyses were made (A), filtration of particles from the leachate solution is not explicit but supposed (sF), plastic particles were rinsed before being used (R) or the external surface of the foam was cut in order to test only the interior (C). Polymers are: Acrylonitrile butadiene styrene (ABS), car tire rubber (CTR), polycarbonate (PC), Polyethylene (PE), high density polyethylene (HDPE), low density polyethylene (LDPE), polyester (PES), polyethylene terephthalate (PET), phenol-formaldehyde (PFA), polyamide (PLA), polymethyl methacrylate (PMMA), polypropylene (PP), polystyrene (PS), expended polystyrene (EPS), polyurethane (PUR), polyvinyl chloride (PVC), styrene-butadiene rubber (SBR), tire wear particle (TWP)

Group	Phylum	Species	Polymer type	Leaching time (h)	Concentration	Exposure time (h)	Effects	Result	Ref	Note
Unicellular	Cyanobacteria	*Plochlorococcus* sp.	HDPE *grossery bag*	120	1.6 - 25 mg/mL	3 - 72	Population growth Photophysiology Oxygen production	↓ ↓ ↓	Tetu et al., 2019	A
			HDPE *grossery bag*	120	3.125 - 25 mg/mL	3 - 48	Population growth Photophysiology Oxygen production	↓ ↓ ↓	Sarker et al., 2020	A, R
			HDPE *grossery bag*	120	6.25 - 12.5 mg/mL	3 - 48	Population growth Photophysiology Oxygen production	↓ ↓ ↓	Sarker et al., 2020	A
			PVC *matting*	120	0.125 - 5 mg/mL	3 - 72	Population growth Photophysiology Oxygen production	↓ ↓ ↓	Tetu et al., 2019	A
			PVC *matting*	120	0.5 - 1 mg/mL	3 - 48	Population growth Photophysiology Oxygen production	↓ ↓ ↓	Sarker et al., 2020	A
			PVC *matting*	120	0.5 - 25 mg/mL	3 - 48	Population growth Photophysiology Oxygen production	↓ ↓ ↓	Sarker et al., 2020	A, R
	Proteobacteria	*Vibrio fischeri*	PET *powder*	72	0.09 mg/mL	0.08 - 0.5	Percentage of natural bioluminescence	↑	Piccardo et al., 2020	A
	Diatom	*Phaeodactylum tricornutum*	PET *powder*	72	0.1 mg/mL	48 - 72	Growth	↑	Piccardo et al., 2020	A
		Skeletonema costatum	CTR *derived granulates*	336	4.8 - 80 mg/mL	0 - 72	Growth	↓	Capolupo et al., 2020	A
			PP	336	4.8 - 80 mg/mL	0 - 72	Growth	↓	Capolupo et al., 2020	A
			PS	336	4.8 - 80 mg/mL	0 - 72	Growth	↓	Capolupo et al., 2020	A
			PVC	336	4.8 - 80 mg/mL	0 - 72	Growth	↓	Capolupo et al., 2020	A

			PET	336	4.8 - 80 mg/mL	0 - 72	Growth	=	Capolupo et al., 2020	A
	Foraminifera	*Haynesina germanica*	PP *pellets*	24	20 - 200 mL/L	10	Locomotion speed Fractal dimension Respiration rate	= = =	Langlet et al., 2020	
	Chlorophyte	*Dunaliella salina*	EPS	672	2 mg /mL or 10 - 30 spheres / 50mL	168	Photosynthetic activity	↑ (fragments < 2mm)	Chae et al., 2020	A
		Dunaliella tertiolecta	PE *pellets*	24	3.1 - 100 mg/mL	24 - 72	Growth inhibition ROS production DNA damages	↑ ↑ ↑	Schiavo et al., 2020	A
			PP *pellets*	24	3.1 - 100 mg/mL	24 - 72	Growth inhibition ROS production DNA damages	↑ ↑ ↑	Schiavo et al., 2020	A
			PS *pellets*	24	3.1 - 100 mg/mL	24 - 72	Growth inhibition ROS production DNA damages	↑ ↑ ↑	Schiavo et al., 2020	A
		Scenedesmus rubescens	EPS	672	2 mg/mL or 10 - 30 spheres / 50mL	168	Photosynthetic activity	↑ (fragments < 2mm and 7-10mm spheres)	Chae et al., 2020	A
		Chlorella saccharophila	EPS	672	2 mg/mL 10 - 30 spheres / 50mL	168	Photosynthetic activity	↑	Chae et al., 2020	A
		Stichococcus bacillaris	EPS	672	2 mg/mL 10 - 30 spheres / 50mL	168	Photosynthetic activity	↑	Chae et al., 2020	A
Pluri-cellular	Cnidaria	*Aurelia* sp	Mixture *Beached MPs*	24	0.033 - 1 mg/mL	24 - 48	Immobility of ephyrae Alteration of frequency of pulsation	= ↑	Cormier et al., 2021	A

		Mixture *Beached MPs*	24	0.033 - 1 mg/mL	24 - 48	Immobility of ephyrae Alteration of frequency of pulsation	= ↑	Cormier et al., 2021	A
		PE *pellets*	24	0.033 - 1 mg/mL	24 - 48	Immobility of ephyrae Alteration of frequency of pulsation	= ↑	Cormier et al., 2021	A
	Stylophora pistillata	PS *foam*	504	0.6 mg/mL	120	Photosynthetic activity Respiration Symbiont density Pigmentation	= = = =	Aminot et al., 2020	A, C
Crustacea	*Amphibalanus amphitrite*	PC *recyclable items*	24	0.004 - 0.5 m^2/L	24	Nauplii mortality Settlement	↑ ↓	Li et al., 2016	A, sF
		HDPE *recyclable items*	24	0.004 - 0.5 m^2/L	24	Nauplii mortality Settlement	↑ ↓	Li et al., 2016	A, sF
		LDPE *recyclable items*	24	0.004 - 0.5 m^2/L	24	Nauplii mortality Settlement	↑ ↓	Li et al., 2016	A, sF
		PET *recyclable items*	24	0.004 - 0.5 m^2/L	24	Nauplii mortality Settlement	↑ ↓	Li et al., 2016	A, sF
		PP *recyclable items*	24	0.004 - 0.5 m^2/L	24	Nauplii mortality Settlement	↑ ↓	Li et al., 2016	A, sF
		PS *recyclable items*	24	0.004 - 0.5 m^2/L	24	Nauplii mortality Settlement	↑ ↓	Li et al., 2016	A, sF
		PVC *recyclable items*	24	0.004 - 0.5 m^2/L	24	Nauplii mortality Settlement	↑ ↓	Li et al., 2016	A, sF
	Artemia sp.	Bio-PFA *foam*	24	10 - 50 mg/mL	24	Nauplii mortality	↑	Trestrail et al., 2020	A
		PFA *foam*	24	10 - 50 mg/mL	24	Nauplii mortality	↑	Trestrail et al., 2020	A
	Limnocalanus macrurus	LDPE *virgin granules*	168	30 mg/mL	72	Mortality Impaired swimming behavior	= =	Lehtiniemi et al., 2021	A
		LDPE	168	7.5 - 30 mg/mL	72	Mortality Impaired swimming behavior	↑ =	Lehtiniemi et al., 2021	A

	recycled frozen vegetable package containing food residues							
	LDPE *recycled frozen vegetable package*	168	30 mg/mL	72	Mortality Impaired swimming behavior	= =	Lehtiniemi et al., 2021	A, R
	SBR *from artificial turf field*	168	30 mg/mL	72	Mortality Impaired swimming behavior	= =	Lehtiniemi et al., 2021	A
	SBR *from recycling factory*	168	30 mg/mL	72	Mortality Impaired swimming behavior	= =	Lehtiniemi et al., 2021	A
Nitocra spinipes	Bio-PES (50% corn starch and 50% aliphatic polyester) *biodegradable garbage bag*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015	
	Bio-PES (50% corn starch and 50% aliphatic polyester) *biodegradable garbage bag*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015	
	Bio-PET *soda bottle*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	R
	Bio-PET *soda bottle*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	R
	HDPE *garbage bag*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
	HDPE *garbage bag*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
	LDPE *Q-tips box*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
	LDPE *Q-tips box*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
	PE	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021	

glove package							
PE *glove package*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021	
PE *beached glove package*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021	
PE *pellets*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021	
PE *pellets*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021	
PE *pellets*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021	
PE *pellets firstly naturally weathered*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021	
PET *liquid soap bottle*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	R
PET *liquid soap bottle*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	R
PET *drinking bottle*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021	
PET *drinking bottle*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021	
PET *drinking bottle*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021	
PET *beached drinking bottle*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021	
PET *pellets*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021	
PET *pellets*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021	
CTR	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015	R
CTR	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015	R
PLA *3D printer plastic*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	

PLA *3D printer plastic*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
PP *DVD case*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
PP *DVD case*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
PP *food packaging*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021
PP *food packaging*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021
PP *food packaging*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PP *food packaging*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PP *flower pot*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021
PP *flower pot*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021
PP *flower pot*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PP *flower pot*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PP *pellets*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021
PP *pellets*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021
PP *pellets*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PP *pellets firstly naturally weathered*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PS *cup*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
PS *cup*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
PS *pellets*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021

PS *pellets*	120	10 - 100 mg/mL	96	Mortality	=	Gewert et al., 2021
PS *pellets*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PS *pellets firstly naturally weathered*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PS *keyboard*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PS *keyboard*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PUR *phone cover*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
PUR *phone cover*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
PVC *garden hose*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
PVC *garden hose*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
PVC *costume packaging*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
PVC *costume packaging*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
PVC *cable insulation*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
PVC *cable insulation*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
PVC *glove*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PVC *glove*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PVC *glove*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
PVC *beached glove*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
Unknown *(mainly PVC)*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021
Unknown *(mainly PVC)*	120	10 - 100 mg/mL	96	Mortality	↑	Gewert et al., 2021

Unknown *toothbrush*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *toothbrush*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *shoe sole packaging*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *shoe sole packaging*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *computer housing (probably ABS/PC)*	72	100 mg/mL	96	Mortality	↓	Bejgarn et al., 2015
Unknown *computer housing (probably ABS/PC)*	72	100 mg/mL	96	Mortality	↓	Bejgarn et al., 2015
Unknown *toy*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *toy*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *shower curtain*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *shower curtain*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015
Unknown *car dashboard from 1983 (probably ABS/PC)*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
Unknown *car dashboard from 1983 (probably ABS/PC)*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015
Unknown *flyswatter packaging (probably PVC)*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015

	Unknown *flyswatter packaging (probably PVC)*	72	100 mg/mL	96	Mortality	↑	Bejgarn et al., 2015	
	Unknown *wire harness connector*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
	Unknown *wire harness connector*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
	Unknown *toothbrush cover (probably PVC)*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
	Unknown *toothbrush cover (probably PVC)*	72	100 mg/mL	96	Mortality	=	Bejgarn et al., 2015	
Uca pugilator	Curbside tailing + PS recycled	24 - 72	piece of 4x2x0.5cm in 100mL	168	Growth Ecdysis time	↑ ↑	Weis et al., 1992	A
Acartia tonsa	TWP *artificial turf*	Unknown	10000 particles / L	24	Fecal pellet production Egg production Hatching	↑ (low food concentrati on) ↑ (low food concentrati on) =	Koski et al., 2021	A
	TWP *old tire*	Unknown	10000 particles / L	24	Fecal pellet production Egg production Hatching	= = =	Koski et al., 2021	A
	TWP *new tire*	Unknown	10000 particles / L	24	Fecal pellet production Egg production Hatching	= = =	Koski et al., 2021	A
Temora longicornis	TWP *old tire*	Unknown	10000 particles / L	24	Fecal pellet production Egg production	↑ =	Koski et al., 2021	A

		TWP *new tire*	Unknown	10000 particles / L	24	Fecal pellet production Egg production	= =	Koski et al., 2021	A
Echinodermata	*Arbacia punctulata*	Curbside tailing + PS recycled	24 - 72	piece of 1*1*0.5cm in 12mL	1 - 504	Egg fertilization Larval growth	= ↓	Weis et al., 1992	A
	Lytechinus variegatus	PE *pellets*	24	0.25 mL/mL	24	Anormalous development	↑	Nobre et al., 2015	
		Unknown *beached pellets*	24	0.25 mL/mL	24	Anormalous development	=	Nobre et al., 2015	
	Paracentrotus lividus	Mixture *beached MP from Marie-Galante Island*	24	0.333 - 10 mg/mL	48	Larval length	↑	Cormier et al., 2021	A
		Mixture *beached MP from Petit-Bourg*	24	0.333 - 10 mg/mL	48	Larval length	↑	Cormier et al., 2021	A
		PE *pellets*	24	0.333 - 10 mg/mL	48	Larval length	↑	Cormier et al., 2021	A
		HDPE *fluff*	1440	10^3 - 10^5 microsphere/mL	48h	% of pluteus larval abnormality	↑ (except for 0.5 mg/mL)	Martinez-Gomez et al., 2017	
		LDPE *additive-free virgin granules*	72	100 mL/L	24 – 48	Embryonic development delay Phenotypic abnormalities	= =	Rendell-Bhatti et al., 2021	A
		PMMA *pellets*	720	0.01 mg/mL	1	% fertilized eggs Developmental defects	= ↑ (particles < 20µm)	Thomas et al., 2020	
		PS *microspheres*	1440	10^3 - 10^5 microsphere/mL	48h	% of pluteus larval abnormality Larval growth	↑ =	Martinez-Gomez et al., 2017	
		PS *pellets*	720	0.01 mg/mL	1	% fertilized eggs Developmental deffect	= =	Thomas et al., 2020	
		PVC *blue toy*	24	1 - 33 mg/mL	48	Larval growth	↓	Oliviero et al., 2019	A
		PVC *green toy*	24	1 - 33 mg/mL	48	Larval growth	↓	Oliviero et al., 2019	A

		PVC *orange toy*	24	1 - 33 mg/mL	48	Larval growth	↓	Oliviero et al., 2019	A
		PVC *MP*	24	1 - 33 mg/mL	48	Larval growth	=	Oliviero et al., 2019	A
		PVC *nurdles*	72	100 mL/L	24 – 48	Embryonic development delay Phenotypic abnormalities	↑ ↑	Rendell-Bhatti et al., 2021	A
		Mixture *beached biobeads (mainly PE)*	72	100 mL/L	24 – 48	Embryonic development delay Phenotypic abnormalities	↑ ↑	Rendell-Bhatti et al., 2021	A
		Mixture *beached nurdles (mainly PE)*	72	100 mL/L	24 – 48	Embryonic development delay Phenotypic abnormalities	↑ ↑	Rendell-Bhatti et al., 2021	A
		PET *powder*	72	0.1 mg/mL	72	Abnormal larvae	↑ (particles > 60µm)	Piccardo et al., 2020	
Mollusca Bivalve	*Choromytilus meridionalis*	PP *pellets*	24	20mL of pellets / L	8	% of aggregated mussels Time to aggregate Crawling distance Byssal thread production	↑ ↑ ↑ =	Seuront et al., 2021	sF
	Meretrix meretrix	PE *bags*	48	0.05 - 10 mg/mL	1 - 72	Fertilization Deformity of larvae Shell heigth of larvae Mortality of larvae	= ↑ ↓ ↑	Ke et al., 2019	A
	Mytilus edulis	PP *pellets*	24	20mL of pellets / L	8	% of aggregated mussels Time to aggregate Crawling distance Byssal thread production	↑ ↑ ↑ =	Seuront et al., 2021	sF
	Mytilus galloprovincialis	PET	336	0.48 - 80 mg/mL	1.5 - 144	Lysosomal membrane stability Gamete fertilization Embryonis development Survival	= ↓ ↓ =	Capolupo et al., 2020	A

PET	336	0.08 mg/mL	168	Lysosomal membrane stability Unsatured neutral lipid content LYS/CYT Lipofuscin content Malondialdehyde content GST activity in digestive gland GST activity in gills CAT in digestive glands and gills Acetylcholinesterase content Metallothionein content	= ↑ = = ↑ = ↑ = = =	Capolupo et al., 2021	A
PFA *foam*	24	1 mg/mL	72	Catalase activity Glutathione S-tranferase activity Acetylcholinesterase activity Malondialdehyde content	= = = =	Trestrail et al., 2020	A
PP	336	0.48 - 80 mg/mL	1.5 - 144	Lysosomal membrane stability Gamete fertilization Embryonis development Survival	↓ ↓ ↓ ↓	Capolupo et al., 2020	A
PP	336	0.08 mg/mL	168	Lysosomal membrane stability Unsatured neutral lipid content LYS/CYT Lipofuscin content Malondialdehyde content GST activity in digestive gland GST activity in gills CAT in digestive glands and gills Acetylcholinesterase content Metallothionein content	↓ ↑ ↑ ↑ ↑ ↑ ↑ = = =	Capolupo et al., 2021	A
PP *pellets*	24	20mL of pellets / L	8	% of aggregated mussels Time to aggregate Crawling distance Byssal thread production	= = = ↑	Seuront et al., 2021	sF
PS	336	0.48 - 80 mg/mL	1.5 - 144	Lysosomal membrane stability Gamete fertilization Embryonis development Survival	↓ ↓ ↓ =	Capolupo et al., 2020	A

PS	336	0.08 mg/mL	168	Lysosomal membrane stability	↓	Capolupo et al., 2021	A
				Unsatured neutral lipid content	↑		
				LYS/CYT	=		
				Lipofuscin content	=		
				Malondialdehyde content	↑		
				GST activity in digestive gland	=		
				GST activity in gills	=		
				CAT in digestive glands and gills	=		
				Acetylcholinesterase content	=		
				Metallothionein content	=		
PVC	336	0.48 - 80 mg/mL	1.5 - 144	Lysosomal membrane stability	↓	Capolupo et al., 2020	A
				Gamete fertilization	↓		
				Embryonis development	↓		
				Survival	↓		
PVC	336	0.08 mg/mL	168	Lysosomal membrane stability	↓	Capolupo et al., 2021	A
				Unsatured neutral lipid content	↑		
				LYS/CYT	↑		
				Lipofuscin content	↑		
				Malondialdehyde content	=		
				GST activity in digestive gland	=		
				GST activity in gills	=		
				CAT in digestive glands and gills	=		
				Acetylcholinesterase content	↓		
				Metallothionein content	=		
CTR *derived granulates*	336	0.48 - 80 mg/mL	1.5 - 144	Lysosomal membrane stability	↓	Capolupo et al., 2020	A
				Gamete fertilization	↓		
				Embryonis development	↓		
				Survival	↓		

		CTR *derived granulates*	336	0.08 mg/mL	168	Lysosomal membrane stability Unsatured neutral lipid content LYS/CYT Lipofuscin content Malondialdehyde content GST activity in digestive gland GST activity in gills CAT in digestive glands and gills Acetylcholinesterase content Metallothionein content	↓ ↑ ↑ ↑ = = = = = =	Capolupo et al., 2021	A
	Perna perna	PP *pellets*	24	20mL of pellets / L	8	% of aggregated mussels Time to aggregate Crawling distance Byssal thread production	= = = ↑	Seuront et al., 2021	sF
	Pinctada margaritifera	PP + PE *ropes*	24 - 120	0.1 - 100 mg/mL	24 - 48	Embryonic development Mortality	↓ ↑	Gardon et al., 2020	A, R
	Pinctada margaritifera	PP + PE *ropes*	24 - 120	0.1 - 100 mg/mL	24 - 48	Embryonic development Mortality	↓ ↑	Gardon et al., 2020	A
Mollusca Gastropod	*Littorina littorea*	PS *pellets*	24	20mL/L	1	Vigilance and antipredator behaviors	↓	Seuront, 2018	sF
	Littorina littorea	Unknown *beached pellets*	24	20mL/L	1	Vigilance and antipredator behaviors	↓	Seuront, 2018	sF

CHAPTER II

LACK OF BEHAVIORAL EFFECT OF SURGICAL MASK LEACHATE ON THE ASIAN SHORE CRAB *HEMIGRAPSUS SANGUINEUS*: IMPLICATIONS FOR INVASION SUCCESS IN POLLUTED COASTAL WATERS

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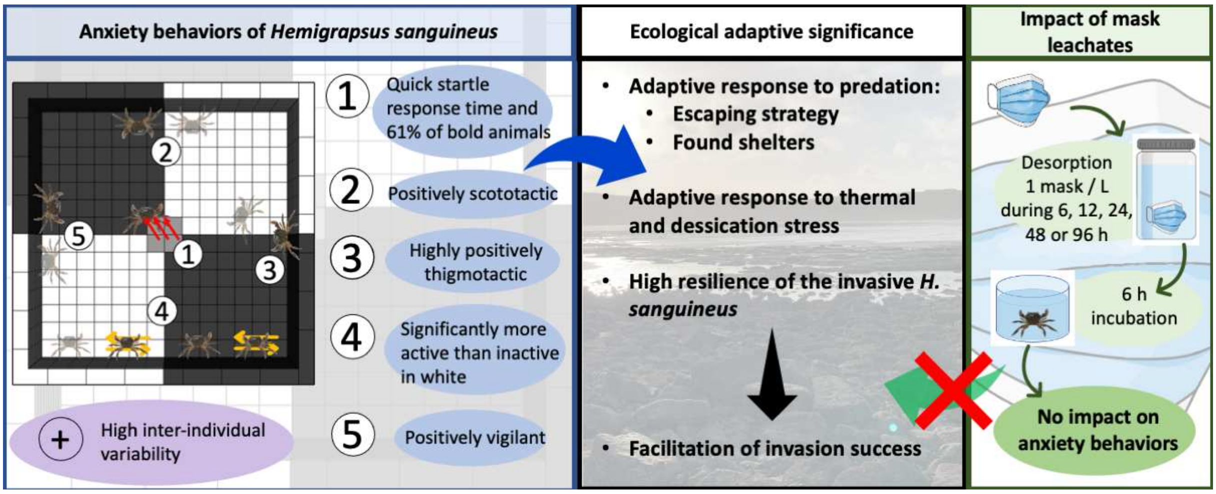
Graphical abstract



Abstract

The COVID-19 pandemic generated a new source of plastic mass pollution, i.e. surgical masks, that preferentially accumulate in intertidal environments. Made of polymers, surgical masks are likely to leach additives and impact local intertidal fauna. As typical endpoints of complex developmental and physiological functions, behavioral properties are non-invasive key variables that are particularly studied in ecotoxicological and pharmacological studies, but have, first and foremost, adaptive ecological significance. In an era of ever-growing plastic pollution, this study focused on anxiety behaviors, i.e. startle response, scototaxis (i.e. preference for dark or light areas), thigmotaxis (i.e. preference for moving toward or away from physical barriers), vigilance and level of activity, of the invasive shore crab *Hemigrapsus sanguineus* in response to leachate from surgical masks. We first showed that in the absence of mask leachates *H. sanguineus* is characterized by a short startle time, a positive scototaxis, a strong positive thigmotaxis, and an acute vigilance behavior. Specifically, a significantly higher level of activity was observed in white areas, in contrast to the lack of significant differences observed in black areas. Noticeably, the anxiety behaviors of *H. sanguineus* did not significantly differ after a 6-h exposure to leachate solutions of masks incubated in seawater for 6, 12, 24, 48 and 96 hours. In addition, our results were consistently characterized by a high inter-individual variability. This specific feature is discussed as an adaptive behavioral trait, which — through the observed high behavioral flexibility — increases *H. sanguineus* resilience to contaminant exposures and ultimately contribute to its invasion success in anthropogenically-impacted environments.

Keywords: surgical masks, COVID, plastic leachate, scototaxis, thigmotaxis, anxiety behaviors

1. Introduction

Plastic is one of the most ubiquitous and conspicuous sources of pollution of the Anthropocene. The marine environment is particularly impacted by this pollution as, each year, 4 to 12 million tons of plastic reach the oceans (Jambeck et al., 2015) where they accumulate and virtually impact every ecosystem from intertidal areas and estuaries down to the seafloor. Noticeably, plastics act as a source of a range of additives and persistent organic pollutants that are respectively absorbed (i.e. intrinsically bounded to the polymer during the manufacturing process), and adsorbed to the surface of plastic polymers (Delaeter et al., 2022; Hahladakis et al., 2018; Hermabessiere et al., 2017). These chemical compounds can be respectively released into the surrounding environment as primary and secondary leachates, and subsequently lead to a range of detrimental effects; see Delaeter et al. (2022) for a review. Recently, the COVID-19 pandemic led to an unprecedent use of personal protective equipment, in particular surgical masks, that caused a new source of mass pollution (Selvaranjan et al., 2021; Oliveira et al., 2023), resulting in the release of more than 1.5 billion of surgical masks in the oceans in 2020 (Phelps Bondaroff and Cooke, 2020). Mainly made of polyethylene (PE) and polypropylene (PP) (Fadare and Okoffo, 2020), surgical masks are likely to impact the marine environment in similar ways than prevailing plastic wastes. More specifically, beyond their potential physical damages, masks are also likely to release leachates in the environment (e.g. up to 393 μg of heavy metals per litre; Sullivan et al., 2021), potentially causing pernicious chemical damages; see Oliveira et al. (2023) for a review.

At the interface between land and sea, intertidal environments typically accumulate humongous quantities of anthropogenic debris (Browne et al., 2010) and surgical masks are no exception (Akhbarizadeh et al., 2021; De-la-Torre and Aragaw, 2021; Haddad et al., 2021). The anthropogenic pollution issue is particularly relevant in these environments, especially rocky shores, as they provide habitats for a great diversity of species (Thompson et al., 2002). In these environments, crabs are key organisms, especially through their predatory role on invertebrates (Little and Finger, 1990; Raffaelli and Hawkins, 1996) from various trophic levels, as well as a food source for a range of predators (Boudreau and Worm, 2012). They are also involved in competitive interactions for resources and shelter with other species (Boudreau and Worm, 2012; Richards and Cobb, 1986; Rossong et al., 2006). As such, any impact leached chemical compounds may have on crabs are likely to have cascading consequences on the structure and function of benthic intertidal communities.

Being endpoints of many complex developmental and physiological functions, behavior has been suggested to be a powerful contamination biomarker (Zala and Penn, 2004), and changes in animal behavior have been acknowledged as the first responses to anthropogenically-altered environmental conditions (Wong and Candolin, 2015). Behavior, and in particular behavioral adaptability, is especially relevant in intertidal organisms to cope with their typically highly variable environment (Thompson et al., 2002). Scototaxis (i.e. preference for dark or light areas) and thigmotaxis (i.e. preference for moving toward or away from physical barriers) have been suggested to play a key role in habitat selection in decapod crustaceans (e.g. seeking shelter; Antonelli et al., 1999) in which they are also used to assess anxiety-like behaviors (Hamilton et al., 2016). When decapod crustaceans are exposed to chemical compounds, especially antidepressants, both anxiety-like and shelter-seeking behaviors (including locomotion) can be disrupted (Mesquita et al., 2011; Fossat et al., 2014; Hamilton, 2016; Peters et al., 2017). However, the potential ecological impacts of leachates from plastic items in general, and surgical masks in particular, on anxiety-like behaviors have yet to be investigated.

Though the ecology of *Hemigrapsus sanguineus* has been well studied (see Epifanio (2013) for a review), relatively little is known about its behavioral ecology. Although the species is known to be negatively phototactic (Spilmont et al., 2015), its scototactic and thigmotactic behaviors have still to be described. They are, however, consistently found under boulders of various sizes, and when dislodged or disturbed, escape and typically seek a physical barrier and/or a dark area (Delaeter, personal observation), which suggests both positive thigmotaxis and scototaxis in their natural environment. Potential disruption of these behaviors, as observed in the sibling *Hemigrapsus orengonensis* under exposure to antidepressant (Peters et al., 2017), may thus have ultimately substantial effects on the survival of the species. In this context, this study aims at (i) characterizing the typical anxiety behaviors of *H. sanguineus*, including scototaxis and thigmotaxis, and (ii) assessing the potential impact of surgical masks' leachate on these behaviors.

2. Materials and methods

2.1. Collection and acclimatation of *Hemigrapsus sanguineus*

Hemigrapsus sanguineus individuals were collected in spring 2021 on the rocky-shore of Fort de Croy, Wimereux, France (50°45'48.9"N, 1°35'59.8"E). Males and non-ovigerous females were sorted on site and subsequently kept in separated running natural seawater aquaria (to prevent reproduction) during a maximum of 72 h before the experiments took place, under conditions of temperature (10°C) and salinity (33 PSU) representative of their habitat when the sampling took place, and in the dark to mimic the intrinsic dimness of their sheltered habitat. Crabs had accessed to crushed mussels in their aquarium before the exposure. They were fed *ad libitum* daily with fresh crushed mussels as described elsewhere (Uguen et al. 2022). Adult individuals (15.6 – 22.6 mm carapace width) were used in the experiments; each individual was used only once, and 40 individuals (i.e. 20 males and 20 females) were tested per treatment.

2.2. Experimental set-up

The behavior of *H. sanguineus* was assessed using a purpose-designed LEGO® Bricks set-up composed of a square arena (side interior dimension: 22.3 cm, height: 7.7 cm; Fig. 1) made of an alternance of white and black tiles that were surrounded by matching color walls (Fig. 1). This set-up was specifically designed to allow the assessment of both the preference for dark *vs.* white areas (i.e. scototaxis) and the response to a physical barrier (i.e. thigmotaxis). We also used a removable grey 'acclimatization cage' (side interior dimension: 3.2 cm, height: 8 cm; Fig. 1) which was consistently held on a set of 4 grey tiles located in the center of the experimental arena (Fig. 1). Two similar arenas were consistently simultaneously used. Though LEGO® Bricks are chemically inert (Lind et al., 2014), we used them without seawater to avoid any potential leaching of additives. A wooden board overlooked the arenas to prevent direct light on the experimental set-up and also served as a camera holder.

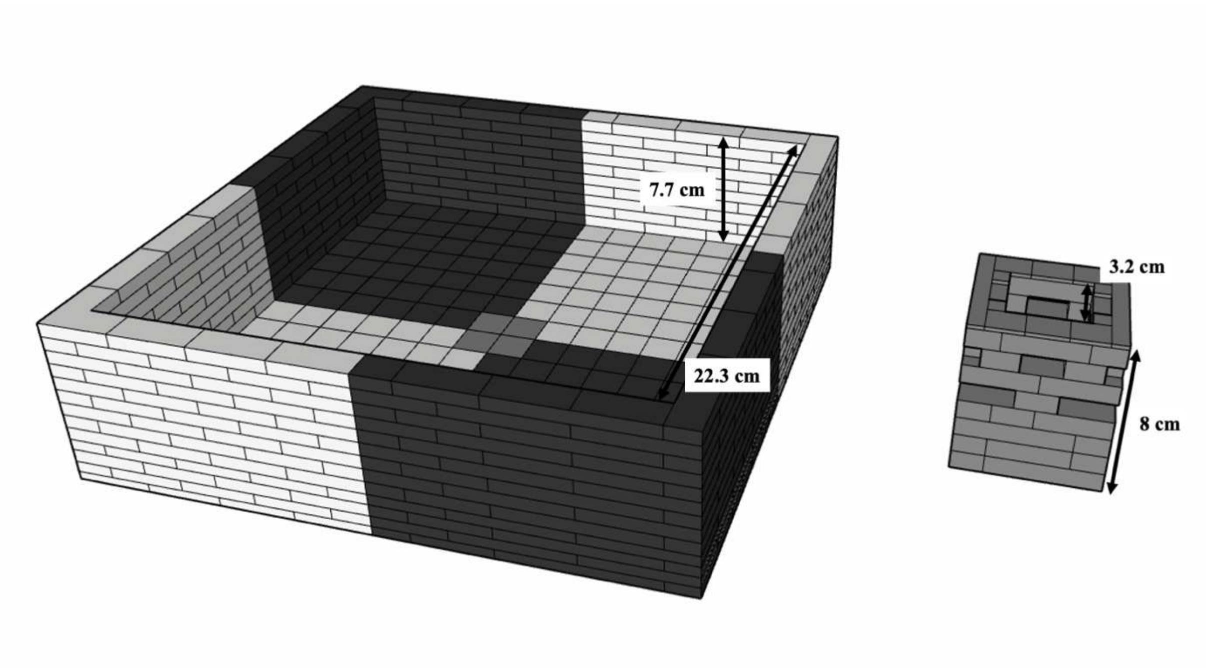


Figure 3. 3D schematic of the LEGO ® Bricks experimental arena (left) and 'acclimatization cage' (right) used to test the anxiety behaviors of *Hemigrapsus sanguineus*. The alternance of white and black squares are surrounded by matching color walls, which respectively allow to assess the preference for dark vs. white areas (i.e. scototaxis) and the response to a physical barrier (i.e. thigmotaxis). The 'acclimatization cage' is placed on the grey central square of the experimental arena and allow to assess the startle response.

2.3. Chemical assessment of mask polymer and additives composition

To identify the polymeric components of the masks (Type IIR Blue, LyncMed Medical technology Co. Ltd., Beijing, China), we conducted Fourier-transform infrared (FTIR) microspectroscopy in attenuated total reflectance (ATR) mode on the three layers composing the masks as well as on the ear band and the nose filter. Micro Tip ATR was placed in contact with the sample to record the spectra, with air as the background spectrum, and polymer types were identified using a Nicolet iN10 Fourier infrared microspectroscope (Thermal Fisher Scientific Co., USA). All spectra were obtained from 4000 to 600 cm^{-1}. Recorded spectra were compared against commercial FTIR spectral libraries (Hummel Polymer and Additives Library, FBI fibre library and PerkinElmer). The identification of the additives content of the masks was carried out using a CDS Pyroprobe 6150 pyrolyzer (CDS Analytical) in conjunction with a GC-HRMS instrument (GC Trace 1310-MS Orbitrap Q Exactive, Thermo Fisher Scientific). Thermal desorption was performed (350°C) to remove the potential additives from the samples. The samples were then separated using a Restek Rxi-5-MS capillary column (30m length, 0.25 mm inner diameter, 0.25 µm film thickness) with a cross-linked poly 5% diphenyl-95% dimethylsiloxane stationary phase (slip ratio: 1:5). The acquisition was conducted on full-scan (FS) mode (m/z = 30.00000–600.00000) and the resulting chromatograms were analyzed using Xcalibur and TraceFinder software for the identification of organic plastic additives among a selection of additives (i.e. plasticizers, flame retardant, antioxidants and UVs stabilizers). The subsequent identification of the additives was based on their retention times, m/z values, and specific ions, which were compared with the chromatograms obtained from standard solutions of each additive. All masks were from the same production batch.

2.4. Leachate preparation and crab incubation

Mask leachate solutions were prepared by incubating 1 virgin mask in 1 L of natural seawater during 6, 12, 24, 48 or 96 h, in airtight glass jars. Based on previous studies showing both the leaching of a range of chemicals from virgin masks (Oliveira et al., 2003) and the persistence of leaching from plastic items over time (e.g. Aminot et al., 2020; Do et al., 2022; Endo et al., 2013; Liu et al., 2019), increasing incubation duration were hypothesized to lead to higher leachate concentrations. To avoid any potential bias due to variations in the masks' composition, all masks originated from the same production batch. The solutions were consistently homogenized by manually shaking the jar (during 10 sec) at the beginning (to allow the mask to properly be immerged) and at the end (to homogenized the solution before the crabs-exposure pots were filled) of each desorption period. Mask were incubated in a constant-temperature room (13 – 15°C) and in the dark to prevent potential photodegradation. Individual crabs were exposed to mask leachates individually during 6 h (corresponding to their natural immersion duration during high tide at the sampling site) in a circular glass jar 6.8 cm in diameter and 4.6 cm high containing 80 mL of leachate seawater. During incubations, the jars were partially covered to maintained dark conditions and to prevent crabs to escape while allowing air exchanges. A control experiment was conducted by incubating crabs in natural seawater following the same procedure.

2.5. Behavioral assays

After a 6 h exposure, an individual *H. sanguineus* was placed in the acclimatization cage on the grey square located in the center of the experimental arena during 5 min. The acclimatization cage was subsequently removed and the crab was free to move within the arena. The crab behavior was then recorded at a rate of 60 images per second during 10 min using a GoPro Hero 5 (GoPro Inc., San Mateo, California, USA) placed 45 cm above the center of the arena. The experimental arenas were rinsed with freshwater and dried with tissues between two successive experiments to avoid any potential bias due to the presence of interspecific chemical cues.

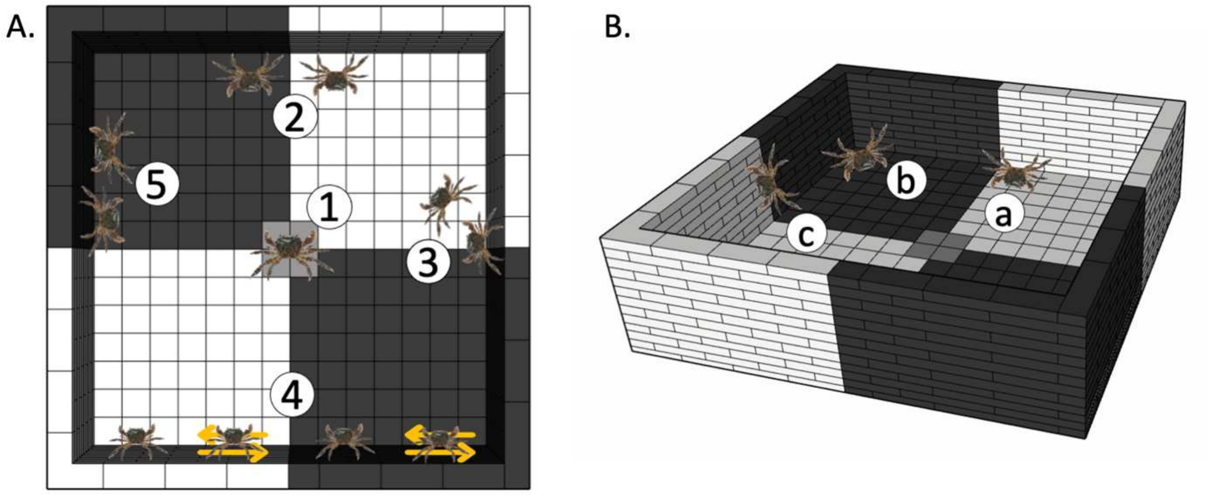


Figure 2. Schematic illustration of (A) the behavioral variables considered for 10 minutes record of the anxiety behaviors of *Hemigrapsus sanguineus*, as (1) the startle response, (2) the scototaxis, (3) the thigmotaxis, (4) the level of activity (arrows represent active individuals) and (5) the vigilance; and (B) the classification of the observed thigmotactic behavior, as walling (i.e. preference for dark or light areas; a), cornering (i.e. preference for moving toward or away from physical barriers; b) and climbing (c).

2.6. Behavioral analysis

H. sanguineus behavior was assessed using 5 anxiety behaviors: the startle response, scototactic and thigmotactic behaviors, level of activity and vigilance (Fig. 2). The startle response describes the behaviors displayed by a crab once the cage is lifted and was first assessed through the startle response time (referred to as startle time hereafter) as the time (in second) spent in the central grey square once the cage is lifted at the beginning of the experiment. Here the startle time is considered as a proxy of the reaction time of a crab following a disturbance (e.g. the removal of its shelter), i.e. a general measure of this species in reacting to stimuli in its environment. We subsequently classified crab personality based on the startle time, i.e. individuals that left the grey central square once the cage was lifted in less and more than 1 second were respectively considered as bold and shy. Scototaxis was evaluated through the percentage of time spent in black (i.e. positive scototaxis) and white (i.e. negative scototaxis) areas of the experiment set-up, once the central grey square was left. The color of the first area chosen by a crab after leaving the central grey square and the color of the first wall reached were considered as scototactic parameters. Thigmotaxis was assessed as the percentage of time a crab spent following a physical barrier, i.e. the walls and corners of the arena. *H. sanguineus* individuals were subsequently classified as walling either actively (i.e. following the walls) or inactively (i.e. staying motionless in contact with the wall), cornering (i.e. staying motionless in a corner) or climbing a wall (without horizontal displacement; Fig. 2). The level of activity was assessed as the percentage of time spent in motion (i.e. active walling) *vs*. motionless (i.e. inactive walling) in each color while exploring the arena. Note that corners were assimilated as shelters, and as such cornering was not considered as an exploratory behavior. Finally, the vigilance was assessed as the percentage of time a crab spent with its eyes oriented toward the center of the arena, while walling or cornering. Note that eyes orientation could not be recorded when an individual was climbing. In addition, the inter-individual variability was measured using the coefficient of variation CV (i.e. the ratio between the standard deviation and the mean), which was estimated for each behavioral parameter. Note that a significant proportion (i.e. 55 to 63%) of climbing individuals felt on their back or escaped from the experimental arena. As such, they were excluded from subsequent analyses to avoid the introduction of uncontrolled biases as the former consistently needed time to get back on their legs and to resume their activity and the latter did not allow for 10 minutes of behavioral observations. As a consequence, the number of crabs actually considered in our analyses ranged between 15 to 19 individuals per treatment.

2.7. Statistical analyses

Because none of our behavioral parameters met the normality assumptions, nonparametric statistics were used throughout this work. First, as no significant differences were observed between males and females' behaviors (Wilcoxon-Mann-Whitney test, $p > 0.05$), males and females were pooled for subsequent analyses. Wilcoxon-Mann-Whitney test (WMW test hereafter) was conducted to assess the potential difference in the time spent active *vs.* inactive while walling in a given color for the control treatment. Kruskal-Wallis test (KW test hereafter) was conducted to assess the presence of potential differences between the control and the 5 treatments. Khi-square (χ^2) test was conducted to assess the potential differences in crab's personality, i.e. the number of bold *vs.* shy individuals, in the 5 treatments compared to the control. All statistical analyses were conducted using the software RStudio (R 4.0.3).

3. Results

3.1. Mask polymeric and additives assessment

ATR-FTIR analysis showed that the three layers of the surgical masks used to prepare our leachate solutions as well as nose filter consistently consisted of polypropylene, while the ear band was made of nylon (Supplementary Materials 2 Fig. S1). The additives assessment revealed that the masks were composed of 5 plasticizers (i.e. the phthalates dibutyl phthalate (DBP), di(2-ethylhexyl)phthalate (DEHP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), dimethyl phthalate (DMP)) and one antioxidant, Irganox® 1081.

3.2. *Hemigrapsus sanguineus* anxiety behaviors

The startle time of *Hemigrapsus sanguineus* exposed to control seawater range from 0 to 118 seconds. More specifically, 61 % (11/18 individuals) and 39% (7/18 individuals) of crabs were respectively bold and shy (Fig. 3A). The vast majority of *H. sanguineus* (16/18 individuals) readily entered a white area after leaving the central grey square, and all of them first reached a black wall irrespective of the color of the first chosen area. Overall, they spent 74.9 ± 31.7%; mean ± standard deviation) of their time in black areas (Fig. 3C), and spent most of their time (99.2 ± 1.8%) in contact with a physical barrier (i.e. positive thigmotaxis; Fig. 3B). Their level of activity, however, differed with the color of the area they were walling in.

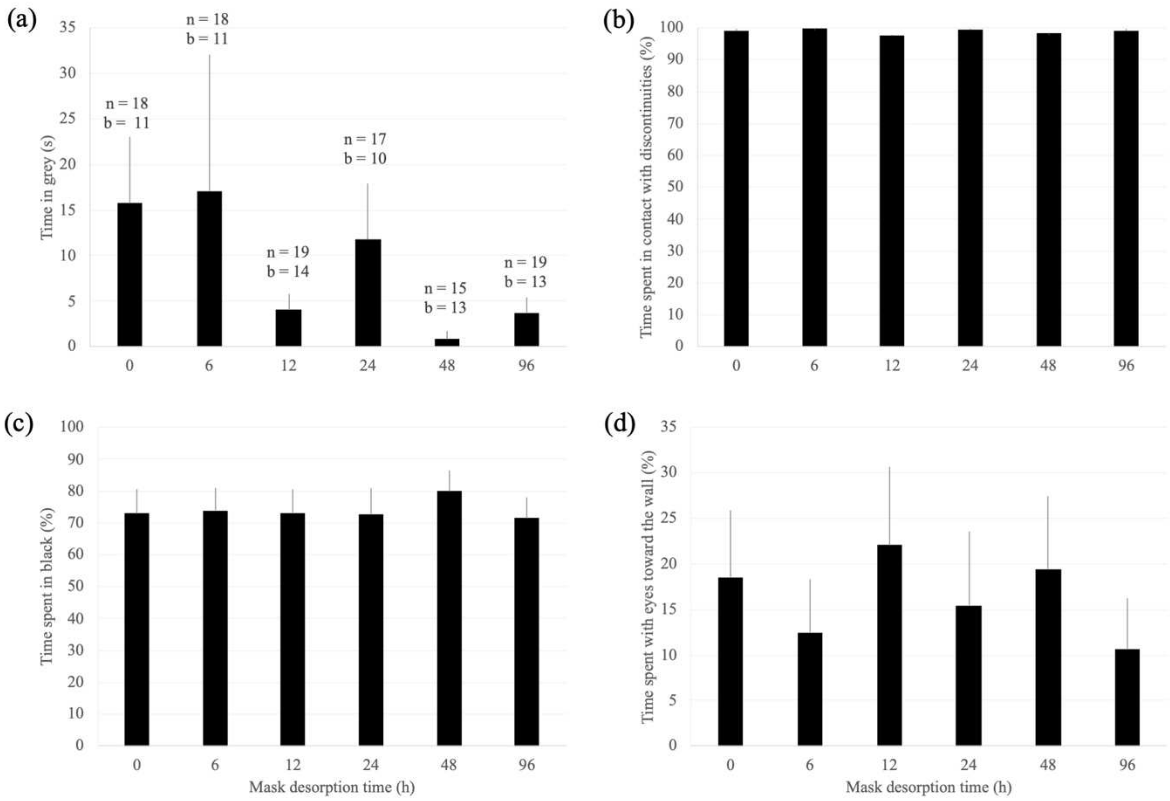


Figure 3. Anxiety behaviors of *Hemigrapsus sanguineus* after the 6h-exposure to virgin surgical mask's leachate (1 mask/L at different desorption time) . The figures represent the average time spent (a) in the central grey square once the cage is lifted (s), i.e. startle time, (b) in contact with discontinuities once the central grey square is left (%), i.e. thigmotaxis, (c) in black areas once the central grey square is left (%), i.e. scototaxis, and (d) with eyes directed toward the wall once when walling and cornering (%), i.e. vigilance, during the 10 min-record depending on the mask's desorption time (h). "0" is the control treatment and corresponds to the basic behaviors as described in 3.1. Errors bars are standard errors, n is the number of analyzed individuals for each treatment (identical for each panel) and b is the number of bold individuals.

Specifically, the proportion of time spent actively and inactively walling in black areas were not significantly different (WMW test, $p > 0.05$). In contrast, crabs were significantly more active than inactive in white areas (WMW test, $p < 0.01$; Fig. 4). Finally, *H. sanguineus* essentially had their eyes directed towards the center of the arena (83.2 ± 31.0%; Fig. 3D). Inter-individual variability, assessed through the coefficient of variation (CV), was high for the startle time and the positive vigilance response (respectively 1.97 and 1.84; Table 1). In comparison, the CV of the scototactic and thigmotactic responses were much lower, i.e. 0.42 and 0.02, respectively.

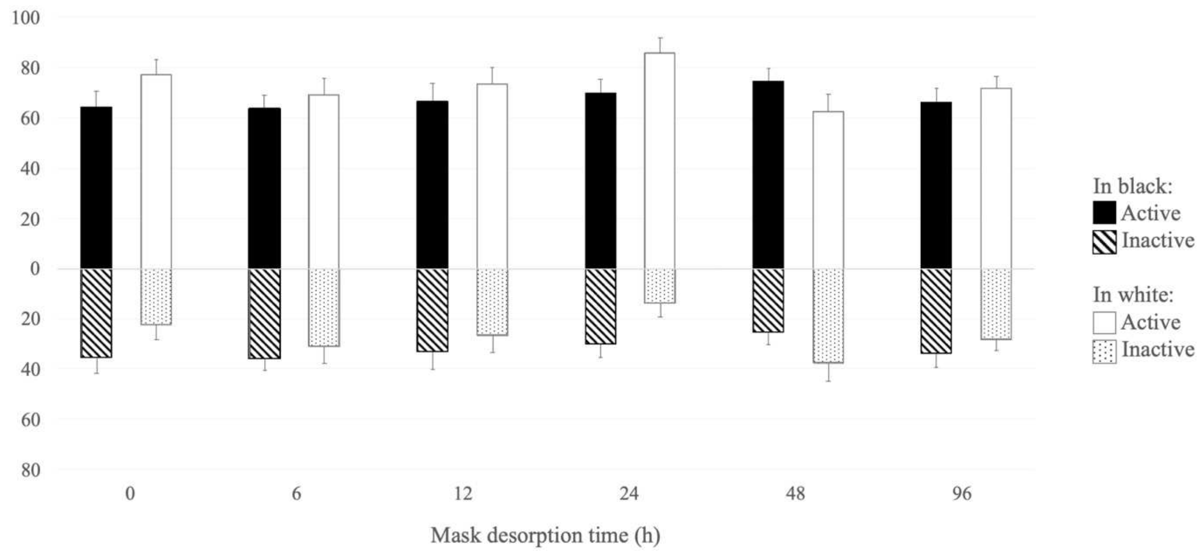


Figure 4. Percentage of the time *Hemigrapsus sanguineus* spent being either active or inactive during the 10 min-record in black or white areas, after 6h-exposure to virgin surgical mask's leachate (1 mask/L); shown as a function of surgical mask incubation time (h). "0" is the control treatment and corresponds to the basic behaviors as described in 3.1. Errors bars are standard errors.

3.3. *Hemigrapsus sanguineus* anxiety behaviors following an exposure to surgical mask leachates

An exposure to mask leachate solutions had no significant effect on startle time regardless of the desorption time (KW test, $p > 0.05$; Fig. 3A). The number of bold individuals was not significantly different from the control (χ^2, $p > 0.05$), except for the 48h-desorption treatment (χ^2 test, $p < 0.05$). Similarly, scototactic response was not impacted by leachate exposure (KW test, $p > 0.05$; Fig. 3B) and individuals still mostly (i.e. 80 to 88.9%) entered a black area when they left the grey central square and always reached a black wall (Table 2). Thigmotactic behaviors were neither modified by leachate exposure (KW test, $p > 0.05$; Fig. 3C). The level of activity in black or white areas was not significantly impacted by the exposure to mask's leachate (KW test, $p > 0.05$). Finally, no significant difference appeared in vigilance behavior, as crab eyes were essentially and consistently directed towards the center of the arena, regardless of the desorption time (KW test, $p > 0.05$; Fig. 3D). In spite of the above-mentioned lack of significant differences between treatments, the behavior of *H. sanguineus* remained characterized by a very high variability (Table 1; Fig. 3), with the noticeable exception of the thigmotactic behavior, which was characterized by a 3- to 10-fold decrease in CV, except for the 96h-desorption treatment (Table 1).

Table 1. Variability in the anxiety behavior of *Hemigrapsus sanguineus* exposed to natural seawater (i.e. "0") or solutions of mask leachate (1 mask/L) desorbed during 6 to 96 h assessed through the coefficient of variation (%) of the startle time (time spent in grey once the cage is lifted), the scototactic response (time spent in black areas once the central grey square is left), the thigmotactic response (time spent in contact with a discontinuity once the central grey square is left) and the vigilance response (time spent with eyes' direction toward the center of the arena while walling or cornering) of "0" is the control treatment. The record of behaviors lasted 10 minutes.

Treatment	Startle time	Scototaxis	Thigmotaxis	Vigilance
0	197	42	1.8	184
6	370	38	0.35	199
12	192	44	0.66	170
24	217	45	0.61	215
48	357	31	0.17	160
96	196	41	1.5	226

Table 2. Percentage of *Hemigrasus sanguineus* individuals observed to first enter a black *vs*. white area and to first touch a black *vs*. white wall after a 6h-exposure to the leachate of mask (1 mask/L) desorbed during 6 – 96h. "0" is the control treatment.

Treatment	Color of the first entered area		Color of the first touched wall	
	Black	White	Black	White
0	88.9	11.1	100	0
6	88.9	11.1	100	0
12	89.5	11.5	100	0
24	88.2	11.8	100	0
48	80	20	100	0
96	89.5	11.5	100	0

4. Discussion

4.1. *Hemigrapsus sanguineus* anxiety behaviors: ecological implications

Our study showed that *H. sanguineus* individuals are characterized by a short startle time and a vast majority of bold individuals, a positive scototaxis, a strong positive thigmotaxis and an acute vigilance behavior. In addition, *H. sanguineus* were significantly consistently more active in white areas. These results are consistent with observations conducted in their natural environment where *H. sanguineus* are commonly found confined under boulders of various sizes, and when dislodged or disturbed escape and seek environments with available, typically shaded and confined, shelter (Delaeter, personal observation).

More specifically, the various behaviors observed in the present work have an adaptive ecological significance. *H. sanguineus* quick startle response is consistent with a quick anti-predation response (Lohrer and Whitlatch, 2002). This behavior is noticeably in sharp contrast with observations conducted on the native green crab *Carcinus maenas* which tends to remain immobile when disturbed rather than fleeing, giving *H. sanguineus* a significant advantage towards tactile predators (Lohrer and Whitlatch, 2002). Note, however, the apparent contradiction between our results (i.e. vast majority of bold individuals with a startle time lower than 1 sec) conducted on well fed individuals and the previously discussed increased boldness propensity in starved individuals (Belgrad et al., 2017) may instead be an indication that boldness is an intrinsic behavioral property of *H. sanguineus* and can be seen as an efficient response to disturbance (e.g. predation) which increases the resilience of the species, and *de facto* facilitate invasion success.

Both positive scototaxis and thigmotaxis have been described as adaptative responses to predation (Low, 1970) — especially for fleeing species (Hughes, 1990) — and/or both thermal and desiccation stress (McGaw, 2001) in crab species such as *Hemigrapsus nudus* (Low, 1970; McGaw, 2001) and *Helice Crassa* (Hughes, 1990). In a slightly different context, thigmotaxis has also been shown to play a pivotal role in facilitating orientation (e.g. exploration, dispersal or foraging) in the tunnelling mud crab *Helice crassa* (Hughes, 1990). In addition, the increased level of activity observed in the present work in white areas is consistent with an adaptation to escape stressful environment (Culumber, 2020).

Even though this behavior has still to be discussed in an ecological context, the acute vigilance observed in the present work in *H. sanguineus* is consistent with previous observation conducted on congeneric species *H. nudus* (Low, 1970). *H. sanguineus* being photophobic (Spilmont et al., 2015), and essentially active at night when they forage in the open areas without exhibiting any thigmotactic behavior (Spilmont and Seuront, unpublished data), the thigmotactic response and related acute vigilance observed in the present work may potentially be related to an endogenous (i.e. circadian) or exogenous rhythm driven by light (Spilmont et al., 2015). Though the resolution of this specific issue lies well beyond the scope of the present study, further investigations are needed to infer if the thigmotactic and vigilance behaviors reported for *H. sanguineus* would remain at night-time and in the dark during day-time.

Finally, *H. sanguineus* were particularly active during the experiments, and a significant amount of them escaped or felt down after trying to climb the walls of the experimental arena in attempts to escape. These observations, beyond the fact that they only allowed us to analyze the behavior of ca. 50% of the tested individuals (i.e. 15-19/40 individuals per treatment), are consistent with the reported hyperactivity (Saxton et al., 2020) and high mobility (Brousseau et al., 2002) of *H. sanguineus*, and the abovementioned adaptation to escape stressful conditions. The observed quick startle response, high vigilance and hyperactivity can be thought as an adaptive strategy to minimize predation risk (Belgrad and Griffen, 2016) while increasing foraging efficiency (Saxton et al., 2020), hence contribute to the invasive success of *H. sanguineus*.

4.2. Mask leachates do not impact anxiety behaviors in *Hemigrapsus sanguineus*

To the best of our knowledge, nothing is known about the potential impact of plastic leachates on crabs; see Delaeter et al. (2022) for a review of the effect of plastic leachates on marine invertebrates. It has, however, been shown that chemicals — including plastic additives — may act at different levels to ultimately impact behavior, through e.g. impacts on enzyme activity (Blewett et al., 2017; Colovic et al., 2013; Nanninga et al., 2020), on endocrine system and hormonal secretion, or on information gathering and decision making (Crump et al., 2020; White and Briffa, 2017). Chemicals can also noticeably reduce detoxification capacity and antioxidative defenses, thus making an organism more vulnerable to stress (Wang et al., 2021). Modifications of anxiety behaviors, i.e. dark preference, level of activity and risk-avoidance, have been observed in crabs when exposed to antidepressant (Hamilton et al., 2016; Peters et al., 2017). Although the behavioral effects of PAHs and heavy metals on crabs have yet to be investigated, it is noticeable that they are commonly used as plastic additives and lead to decrease genetic variability (Fratini et al., 2008), trigger the expression of stress response genes (Baratti et al., 2022) and increase larval mortality (Oliva et al, 2019) in the crab *Pachygrapsus marmoratus*.

Despite the ever-growing body of literature illustrating the impact of plastic in general, and plastic leachate in particular (Delaeter et al., 2022), on the behavior of a range of invertebrates, the present study showed that mask leachates did not significantly impact the anxiety behaviors of *H. sanguineus*. Our chemical analyzes of the composition of masks revealed that they contained various phthalates (i.e. DBP,DEHP, DEP, DIBP and DMP) and one antioxidant (i.e. Irganox® 1080). Phthalates are known as endocrine disruptors even at low concentration, and induced numerous impacts on aquatic organisms, including on behaviors; see Oehlmann et al. (2009) for a review of the impact of phthalates on aquatic organisms. Previous studies revealed impacts of DBP on detoxification and antioxidative defense mechanisms and on the molting of the swimming crab (*Portunus trituberculatus*; He et al., 2022). The absence of any impact of mask leachates on crabs may be related to the acknowledged resistance of *H. sanguineus*, as illustrated by the lack of impairment of their startle response following exposure to a wide range of environmental factors related to the typical gradients characterizing intertidal ecosystems (i.e. temperature, substrate, elevation, time of the day and conspecific density; Saxton et al., 2020) or its resistance to neurotoxins (Yamamori et al., 1992). These observations, as well as the results of the present work, are consistent with behaviors that are specific of resilience to anthropogenic environmental changes (Sih et al., 2011), such as boldness or behavioral flexibility. Though these behaviors have been shown to be characteristic of invasive species and facilitate invasion success (Carere and Gherardi, 2013; Chapple et al., 2012), our results clearly indicate that they may also contribute to the resistance to plastic pollution. Alternatively, the lack of impact on the anxiety behaviors of *H. sanguineus* could be related to the exposure time (i.e. 6 h, duration of immersion during high tide on our study site) that may not be long enough to elicit a behavioral response, irrespective of the desorption time to allow any form of bioaccumulation in *H. sanguineus*. This hypothesis is consistent with results showing that the exposition of *Carcinus maenas* to polystyrene during 5 days led to a bioaccumulation of chemical compounds in crab tissues, but had no impact on their feeding efficiency (Cunningham et al., 2021a). Although the issue lies well beyond the scope of the present study, further experiments are needed to investigate the impact of a chronic exposure to masks' leachates on crab anxiety behaviors. Future studies could also benefit from exploring the effect of exposure on crab's anxiety behaviors by monitoring their behavior during the exposure to gain a more comprehensive understanding of the impact leachates on crabs.

Note, that a recent study conducted by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) suggested that concentration of the chemicals found in masks did not exceed health thresholds (https://www.anses.fr/fr/content/masques-chirurgicaux-pas-de-dépassement-des-seuils-sanitaires-en-contaminants-chimiques; accessed on 12/02/2022). These results are, however, based on the '*compliance with the recommended conditions of use*' which *de facto* do not include the issue of the chemical behavior of surgical masks once released in the ocean. In addition, we analyzed only the additives composition of the masks and the lack of chemical analysis of the leachates content is a clear limitation of this study that will need to be improved in future work.

4.3. Inter-individual variability as an answer to cope with stress

Inter-individual variability has consistently been high in both control seawater and mask leachate treatments, in particular for scototaxis, vigilance and startle time, and was noticeably independent of desorption time (Table 1). Thigmotaxis was, however, characterized by a lower inter-individual variability (Table 1). This observation may suggest that the preference for a contact with a discontinuity is dominant over the preference for dark areas in *H. sanguineus*. Further experiments are, however, needed to confirm this hypothesis, i.e. giving the choice between dark areas with and without discontinuity or light areas with and without discontinuities.

The existence of inter-individual variability also suggests that individuals of the same species are likely to adopt very different traits, or intensities, of a behavior. Specifically, this variability may be related to behavioral plasticity, i.e. a phenomenon that allows for the adoption of a certain intensity of a behavioral trait belonging to a continuum between a maximum and a minimum (Biro et al., 2013; Réale et al., 2007). In the case of anxiety behavior, several continuums have been well studied, e.g. shyness/boldness, aggressiveness, activity, sociability (Réale et al., 2007). Inter-individual variability might also be related to behavioral personality, that is a trait consistently adopted by an individual over time in a given context (Réale et al., 2007; Wolf and Weissing, 2012).

Inter-individual variability is closely related to the environmental stability of a given habitat, with behavioral variability increasing with environmental one (Klopfer and MacArthur, 1960). This feature is consistent with our observations conducted on *H. sanguineus*, an invasive species that proliferates in a temporally and spatially highly complex environment. Inter-individual variability has indeed been shown to contribute to invasion success, i.e. an introduced species would be more likely to expand rapidly if its population is composed of a mixture of different behaviors (Fogarty et al., 2011). Inter-individual variability allows individuals to better cope with environmental changes, hence to improve their fitness through e.g. increased predator avoidance, competitivity for space and resources, and reproduction success and has a pivotal buffering effect at population and ecosystem levels (Wolf and Weissing, 2012). In this context, the absence of any behavioral impact of surgical mask leachates on *H. sanguineus* may be another illustration of the behavioral flexibility of this species to be resilient to contaminant exposures and to be a successful invader in an environment as anthropogenically-impacted and polluted as the eastern English Channel.

5. Conclusion

Our study showed that key behavioral traits of the invasive Asian shore crab *Hemigrapsus sanguineus* — i.e. startle response, both positive scototaxis and thigmotaxis and vigilance — were not impaired following a 6-h exposure to leachates from surgical masks. Instead, *H. sanguineus* were characterized by a high inter-individual variability that we considered as an adaptive behavioral trait, which likely increases *H. sanguineus* resilience to contaminant exposures and ultimately contribute to its invasion success in anthropogenically-impacted environments. We cannot rule out, however, that our short-term incubations — though representative of the immersion time typically experienced by *H. sanguineus* in their tidally-driven environment — may not be representative of the chronic exposure actually encountered in plastic contaminated environments. As such, further work is warranted to decipher the potential effect of repeated exposures to mask leachates in particular and plastic leachates in general on the behavioral traits and cognitive abilities of *H. sanguineus*, which are critical to understand the invasion dynamics in polluted environments.

Acknowledgement

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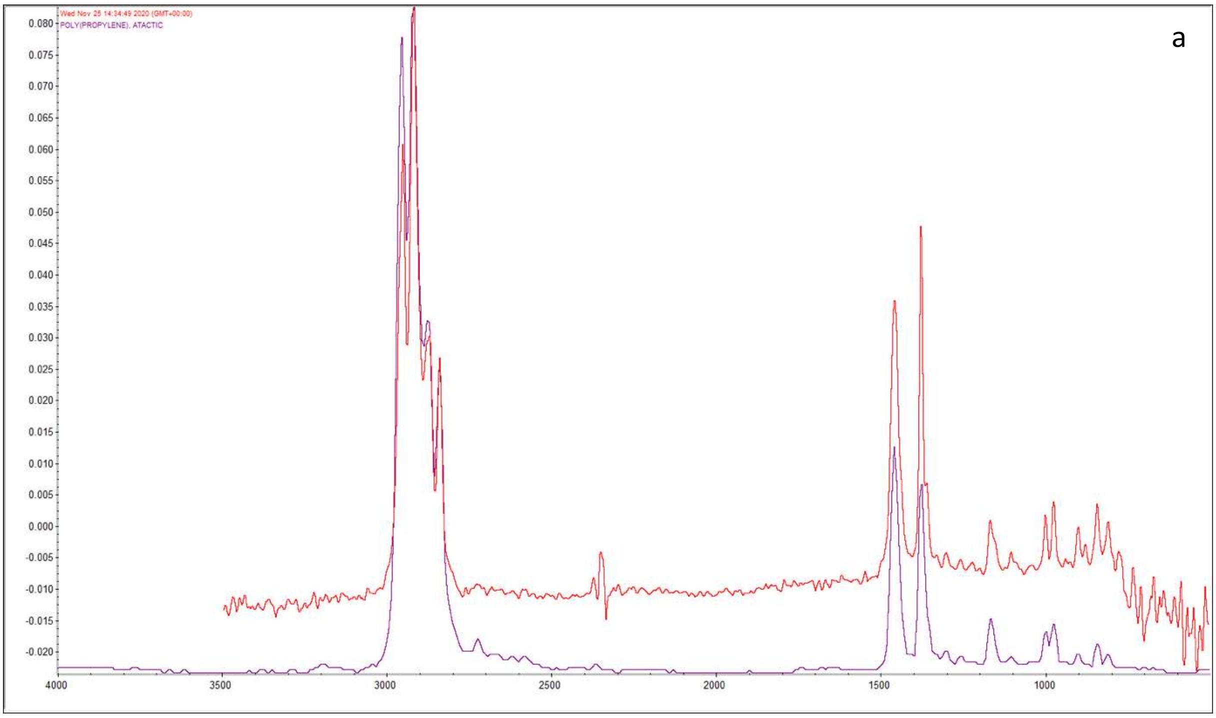
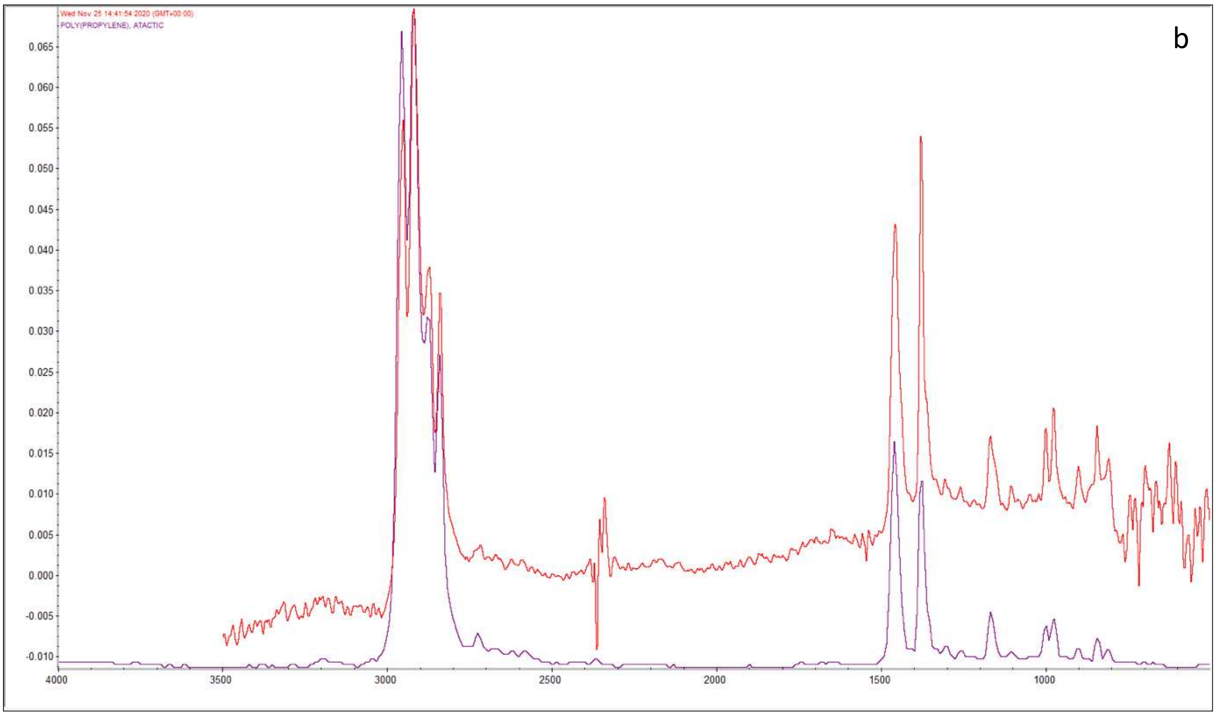
Supplementary Materials 2





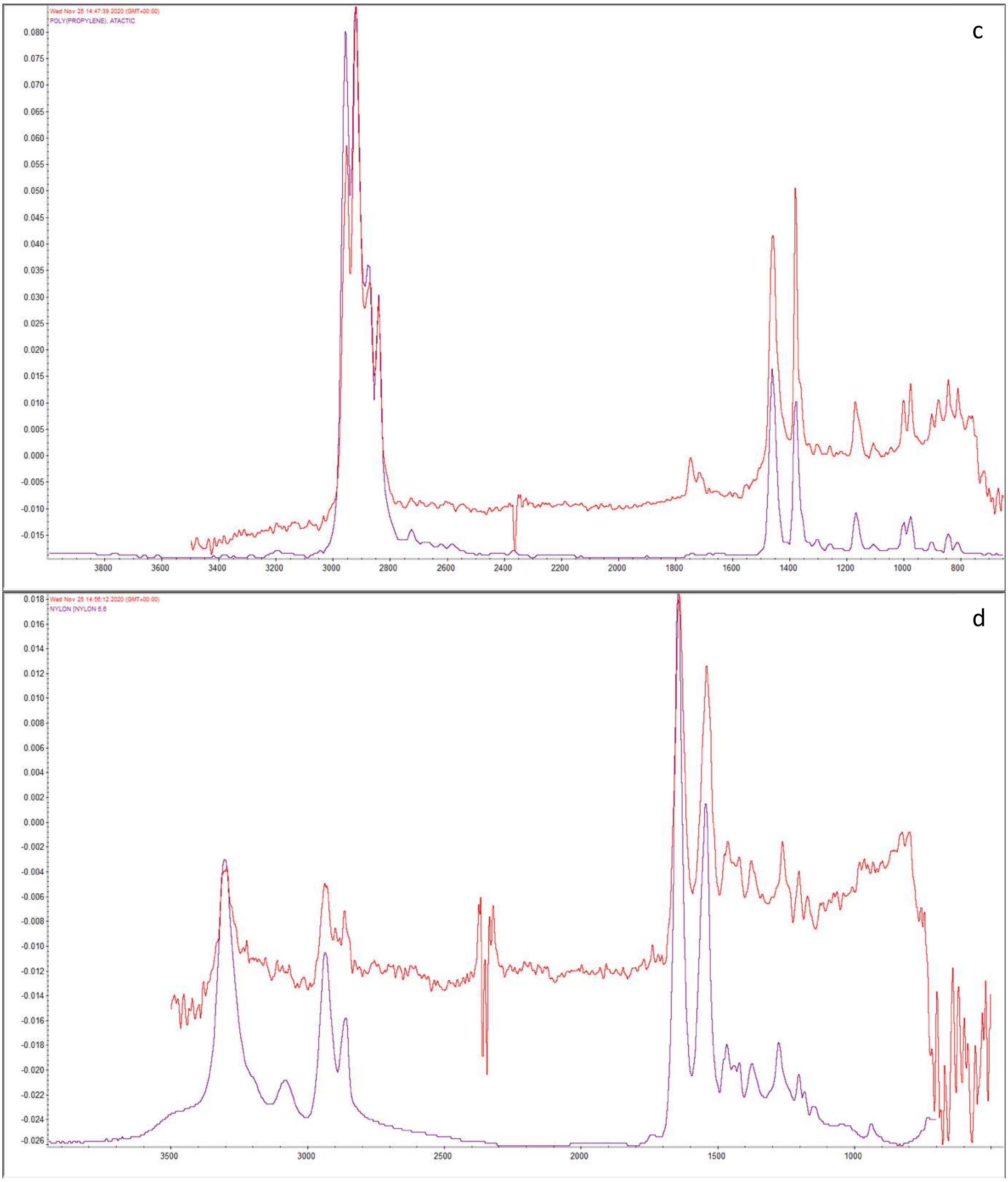
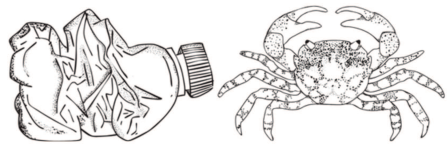


Figure S1. ATR-FTIR spectra for the surgical masks used in the present work. From top to bottom: the three mask layers (a, b & c) and the ear band (d).

CHAPTER III



IMPACTS OF PLASTIC LEACHATES ON THE ANXIETY BEHAVIORS OF THE ASIAN SHORE CRAB *HEMIGRAPSUS SANGUINEUS*.

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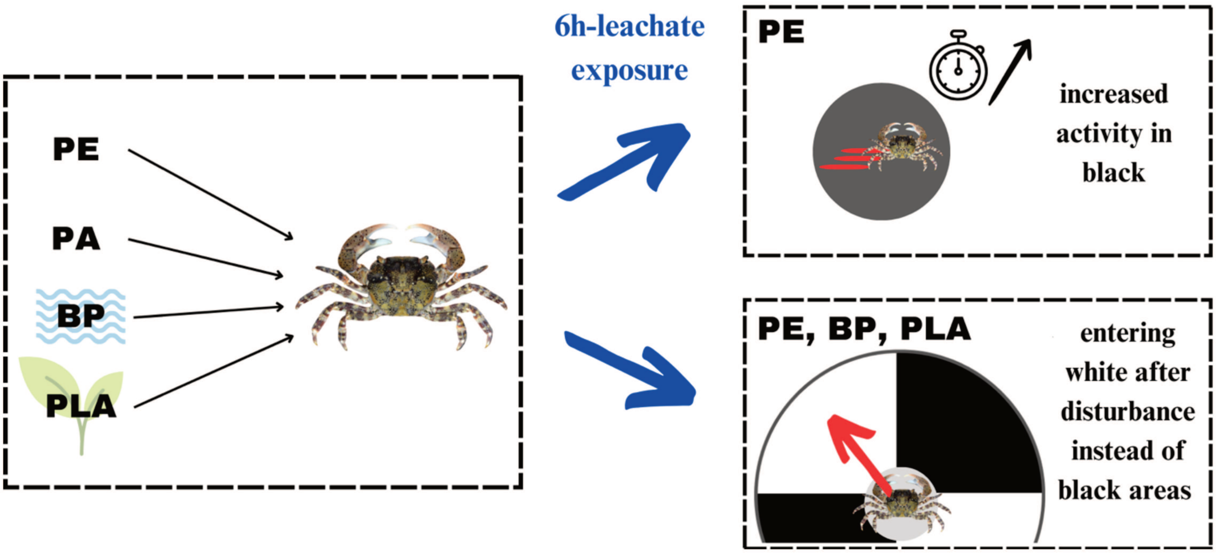
Graphical abstract



Abstract

Intertidal organisms are particularly exposed to plastic pollution with plastic debris comprising up to 95% of litter on shorelines. This pollution is worsened by the leaching of potentially toxic chemicals from plastics into the environment. Plastic leachates can contain additives and pollutants known to be carcinogenic, mutagenic and endocrine disruptors, posing a serious menace to intertidal organisms. Individual behaviors, reflecting complex developmental and physiological processes, are often scrutinized in ecotoxicological studies due to their sensitivity to environmental changes and pollution. In this work, we investigated the anxiety behaviors of the Asian shore crab *Hemigrapsus sanguineus* following an exposure to various plastic leachates, including polyethylene (PE), polyamide (PA), beached pellets (BP) and polylactic acid (PLA, a biobased and biodegradable polymer). We conducted a series of experiments in a specifically designed arena, observing the crabs' behaviors (i.e. startle responses, scototaxis, thigmotaxis, activity level and vigilance behavior) over a 10-minutes period after a 6h exposure to these plastic leachates. Our findings revealed, for the first time, a significant impact on the behaviors of crabs. Following an exposure to PE, BP and PLA leachates, *H. sanguineus* significantly exhibited a preference for entering white over black areas, when the acclimatization cage was removed. Furthermore, exposure to PE leachates lead to a significant increase in the activity level within black areas. Notably, PA leachate did not appear to impact any of the anxiety-related behaviors we examined. This study suggests that plastic leachate exposure may render *H. sanguineus* more vulnerable to predation, possibly through detrimental effects on their metabolism or cognition abilities, and that these impacts are polymer dependent. Additionally, our research highlights the alarming toxicity of bioplastics, often marketed as a solution to plastic pollution, but found to be as toxic as conventionnel fossil fuel ones.

Keywords: plastic leachates, bioplastics, *Hemigrapsus sanguineus*, scototaxis, thigmotaxis, anxiety behaviors

1. Introduction

Plastic pollution is one of the most ubiquitous sources of pollution of the Anthropocene threatening the marine environment. Noticeably, each year an astonishing 4 to 12 million tons of plastic enter the marine environment (Jambeck et al., 2015). Particularly accumulating in intertidal habitats (Browne et al., 2010; Lebreton et al., 2019), plastic debris represent up to 95% of debris on shorelines (Galgani et al., 2015). Beyond their well-documented physical damages (e.g. ingestion, entanglements, habitat destruction; Andrady, 2011; Gall and Thompson, 2015; Uhrin et al., 2014), plastics are also intrinsically harmful through their composition. Manufactured with additives that are not chemically bound to the polymer matrix (Hermabessiere et al., 2017) and prone to accumulate contaminants during their stay in the environment, plastics readily leach a hazardous cocktail of chemicals throughout their lifespan leading to pernicious chemical effects on marine organisms. Noticeably, plastic additives have been identified as carcinogenic, mutagenic or endocrine disruptor (Weis, 2019) causing sub-lethal impairments on e.g. reproduction, embryonic development, cellular integrity, oxidative stress or behaviors across a wide range of marine species (see Delaeter et al. (2022) for a review).

Described as the “first line of defense” (Mench, 1998) in response to changing environments (Wong and Candolin, 2015), individual behavior plays a pivotal role in shaping various ecological processes that influence organisms’ abundance, diversity and extinction. Moreover, behaviors are of utmost importance in species interactions. A change in the behavior of one species can have far-reaching consequences through cascading direct and indirect effects throughout a community at all trophic level (Saaristo et al., 2018; Woodward, 2009). Through its central roles, behavior significantly influence population dynamics and community structure, thereby contributing to ecosystem functioning (Candolin and Rahman, 2023; Candolin and Wong, 2019; Ford et al., 2021; Saaristo et al., 2018) and, ultimately, evolution rates (Duckworth, 2009). Furthermore, behavior proves to be sensitive to environmentally relevant concentrations of contaminants, often more than traditional toxicological endpoints, such as LC50 (Arnold et al., 2014; Little and Finger, 1990; Sih et al., 2010). As a result, behavior is a critical endpoint in anthropogenic ecotoxicological research. Noticeably, anxiety and risk-taking behaviors (directly involved in species interactions) are particularly studied in ecotoxicology studies (e.g. Blewett et al., 2017; Greenshields et al., 2021; Maximino et al., 2010a, 2010b; Prut and Belzung, 2003; White and Briffa, 2017). For instance, serotonin and fluoxetine altered both anxiety-like and shelter-seeking behaviors (including locomotion) in crabs (Hamilton et al., 2016; Mesquita et al., 2011; Peters et al., 2017).

Crabs are widespread in marine habitats, from temperate to tropical and polar environments, playing a key role in trophic interactions. In intertidal environments, crabs act both as predator on invertebrates from various trophic levels (Little and Finger, 1990; Raffaelli and Hawkins, 1996) and as a prey for numerous higher organisms (Boudreau and Worm, 2012). Additionally, they are also involved in competitive interactions for resources and shelter with other species (Richards and Cobb, 1986; Rossong et al., 2006), ultimately shaping the structure and function of benthic intertidal communities (Boudreau and Worm, 2012). Although previous studies have highlighted the effects of plastic particles on different crabs' behaviors, e.g. startle response (Nanninga et al., 2020), defense and attack behaviors (Cunningham et al., 2021b) and shell selection ability (Crump et al., 2023, 2020; McDaid et al., 2023) of the hermit crabs, research on plastic leachates barely exists. The only study existing (to the best of our knowledge) found no impacts of surgical mask leachate (made of PP) on *Hemigrapsus sanguineus* anxiety behaviors (Delaeter et al., 2023). Leachates' toxicity, however, depends on the polymer type and plastic composition, and surgical masks is likely to contain less toxic chemicals than traditional plastic items. In addition, it has, been shown that chemicals potentially released by plastics act at different levels to ultimately impact crabs' behavior, through e.g. impacts on enzyme activity (Blewett et al., 2017), gene expression (Baratti et al., 2022), respiration rate and activity (Greenshields et al., 2021), neurotransmission (Oliva et al., 2019), startle response (i.e. suggested to be due to impairments in information gathering and decision making; White and Briffa, 2017). Therefore, it becomes imperative to conduct further investigations to draw conclusive insights into the influence of plastic leachates on *H. sanguineus* behaviors.

In light of this knowledge gap and the crucial role of crabs in intertidal ecosystems, our study focused on assessing the potential impacts of plastic pellets leachates from both conventional and bioplastics on the anxiety behaviors of *H. sanguineus*.

2. Materials and methods

2.1. Collection of *Hemigrapsus sanguineus*

Hemigrapsus sanguineus individuals were collected in January 2023 on the rocky-shore of Fort de Croy, Wimereux (France, eastern English Channel, 50°45'48.9"N 1°35'59.8"E). Before each experimental trial, males and non-ovigerous females were placed in separate flowing seawater aquariums (L: , l: , H:) overnight, at a temperature representative of their natural habitat at the time of sampling (10 – 12°C), and exposed to natural light. Rocks from the sampling location were added in the aquariums to provide shelter for the crabs.

2.2. Experimental set-up

The experimental set-up for assessing the behavior of *H. sanguineus* was adapted from Delaeter et al. (2023) and consisted of a purpose-designed circular arena (i.e. 22 cm diameter and 11 cm high round glass crystallizer) made of alternating white and black areas, surrounded by matching color walls (Fig. 1). This configuration enabled the evaluation of the crabs' preference for dark *vs.* white areas (i.e. scototactic behaviors) and their response to a physical barrier (i.e. thigmotaxis). The choice of a round arena was deliberate to discourage crabs from staying immobile in a corner ("cornering", not considered as an exploratory behavior; Delaeter et al. 2023). Additionally, a movable ‘acclimatization cage’ (i.e. a PVC pipe; L: 4.5 cm, H: 10 cm), from which the crab was release at the beginning of the assay, was consistently positioned on the central grey circle (Fig. 1)

To ensure optimal condition, the room’s lighting was indirect and arenas were positioned in order to avoid any inside shadows. Three similar arenas were used simultaneously to run the experiments with an overlooking white wooden board used as camera holder. Special attention was given to not visually disturb the crabs during the experiments: the camera holder’s color matched that of the walls and ceiling of the experimental room and the cameras were remotely controlled. Moreover, the assignment and orientation of the arenas were chosen randomly.

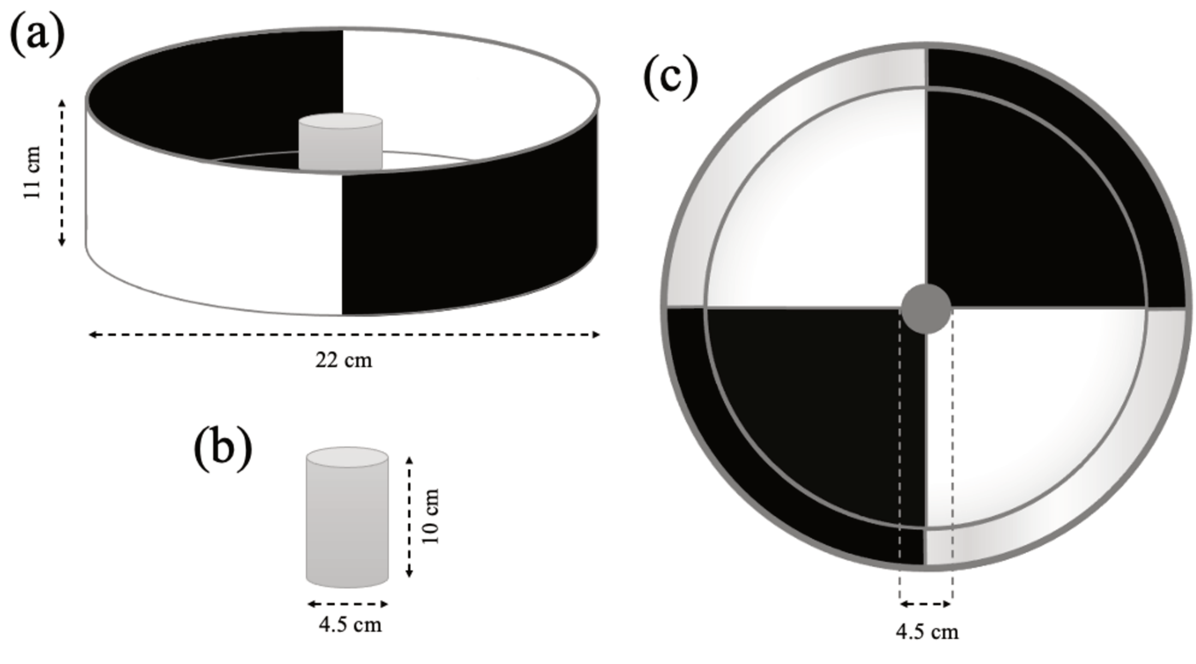


Figure 4. 3D schematic of the experimental arena (a&c) and the acclimatization cage (b): (a) side view of the arena containing the acclimatization cage and (c) top view of the arena. The alternance of white and black areas are surrounded by matching color walls, which respectively allow to assess the preference for dark vs. white areas (i.e. scototaxis) and the response to a physical barrier (i.e. thigmotaxis). The ‘acclimatization cage’ is placed on the grey central square of the experimental arena and allow to assess the startle response.

2.3. Polymer's selection and analyses

Four plastic pellet types were selected for this study: virgin polyethylene (PE, Materialix Ltd.), virgin polyamide (PA, Akulon F136-C1), beached pellets (BP, collected from Neufchâtel-Hardelot beach in France, 50°38'27.2"N, 1°34'37.4"E) and virgin polylactic acid (PLA, NatureWorks LLC, Ingeo™ 4043D). PE, being the most produced polymer in Europe (PlasticsEurope, 2021), is also the most encountered in the field (Erni-Cassola et al., 2019; Mendes et al., 2021). PA, also known as nylon, has been under-represented in the leachate literature compared to its presence in the environment (Delaeter et al., 2022). BP (mainly composed of PE) may have accumulated significant amount of persistent organic pollutants from the environment (Fries and Zarfl, 2012). Finally, PLA is a bio-sourced and biodegradable polymer that ranked among the most produced biopolymer in 2021 (European Bioplastics, 2023).

The identification of the additives content of the plastic pellets was assessed using a CDS Pyroprobe 6150 pyrolyzer (CDS Analytical) in association with a GC-HRMS instrument (GC Trace 1310-MS Orbitrap Q Exactive, Thermo Fisher Scientific). Thermal desorption was performed (350 °C) to remove the potential additives from the samples. The samples were then separated using a Restek Rxi-5-MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with a cross-linked poly 5 % diphenyl-95 % dimethylsiloxane stationary phase (slip ratio: 1:5) and the acquisition was conducted on full-scan (FS) mode (m/z = 30.00000–600.00000). The resulting chromatograms were analyzed using Xcalibur and TraceFinder software for the identification of organic plastic additives among a selection of 57 additives (i.e. plasticizers, flame retardant, antioxidants and UVs stabilizers). The subsequent identification of the additives was based on their retention times, m/z values, and specific ions, which were compared with the chromatograms obtained from standard solutions of each additive.

2.4. Leachate preparation and crab incubation

Due to previous evidence of the insensitivity of *H. sanguineus* to surgical mask leachates (Delaeter et al., 2023), for the present study crabs were exposed to high concentrations of plastic pellets leachates. For PE pellets (4.0 x 2.0 mm, cylindrical shape), the concentration was set at 50 g/L. Subsequently, the concentrations for the other pellet types were determined based on their available exchange surface of the pellets: 50 g/L of PE pellets corresponded to 53.3 g/L of PA (3.0 x 2.2 mm, ellipsoidal shape), 57.4 g/L of BP (3.5 x 3.1 mm, cylindrical shape) and 90.8 g/L of PLA (4.7 x 3.6 mm, ellipsoidal shape).

Pellets were incubated in the dark in 4 L of natural aerated seawater during 24h in temperature-controlled room set to 12°C (i.e. 11.7 – 12.8°C) and aerated using an air pump. A 4 L seawater control solution (without pellets) was incubated under the same conditions. After the 24-hour incubation period, the pellets were separated from the water using a 2 mm mesh sieve, and the resulting solutions were promptly used for the experiment.

During the experiment, crabs were exposed to plastic leachates individually during 6h (corresponding to their natural immersion duration during high tide at the sampling site) in a glass jar (L: 10 cm, H: 20 cm) containing 200 mL of contaminated seawater. Jars were high enough to prevent individuals to escape while allowing air exchanges and aluminum foil surrounded the pots to avoid visual disturbance. Control experiment was driven by incubating the crabs in natural seawater following the same procedure. Conducted over a period of two weeks, the experiments involved 6 control replicates and 20 plastic leachates replicates per experimental day (i.e. 4 days of experimental exposure, each day being devoted to one polymer type). This approach ensured that the crabs were in a consistent physiological state and exhibited identical behaviors under control conditions, enabling meaningful comparisons between leachate treatments.

2.5. Behavioral assays

After a 6 h exposure, one individual *H. sanguineus* was placed inside the acclimatization cage on the grey circle located in the center of the experimental arena during 5 min. The acclimatization cage was subsequently lifted allowing the crab to move freely within the arena. The crab's behavior was then recorded at a rate of 60 images per second during 10 min using a GoPro Hero 5 (GoPro Inc., San Mateo, California, USA) positioned 50 cm above the arena's center. To ensure a clean slate for each experiment and prevent any potential bias from interspecific chemical cues, the experimental arenas were thoroughly rinsed with freshwater and dried using tissues between two successive experiments. Each crab (15.21 – 21.54 mm, carapace width) was used only once, and a total of 20 individuals (i.e. 10 males and 10 females) were tested per leachate treatment (i.e. for a total of 104 crabs). After each experimental trail, remaining individuals were returned to the sampling location and a new batch of individuals was collected.

2.6. Behavioral records analysis

H. sanguineus behavior was evaluated using 5 anxiety behaviors, previously described in Delaeter et al. (2023): the startle responses, the scototaxis, the thigmotaxis, the level of activity and the vigilance. The startle responses of a crab refer to its behavior once the cage is lifted. The startle response time (termed "startle time" hereafter) was measured as the time (in seconds) the crab spends in the central grey circle immediately after the cage is lifted at the beginning of the experiment. The startle time is considered as a proxy for the crab's reaction time to disturbances (e.g. the removal of its shelter), allowing classification into bold (startle time < 1s) and shy (startle time ≧ 1s) individuals. The colors of the first area entered after leaving the central grey circle and the first reached wall, in addition with scototaxis (i.e. the time spent in black *vs.* white areas once the grey square was left), were recorded and considered as scototactic parameters. Thigmotaxis was assessed as the percentage of time a crab spent following the walls of the arena. Vigilance was determined by the percentage of time a crab spent with its eyes oriented toward the arena's center during walling. In addition, inter-individual variability was measured using the coefficient of variation CV (i.e. the ratio between the standard deviation and the mean) for each behavioral parameter. The level of activity was assessed as the percentage of time spent in active *vs.* inactive (i.e. motionless along the wall) in each color while exploring the arena.

Since no significant differences were observed between the behaviors of males and females (Wilcoxon-Mann-Whitney test, $p > 0.05$), their data were grouped. Some individuals felt from the acclimatization cage while lifted and were excluded from subsequent analyses since their startle stimuli was different from the others. The final analysis included behaviors of 19 to 20 individuals per leachate treatments and 24 individuals in the control group (see Fig. 2), regardless their sex.

2.7. Statistical analyses

Because none of our behavioral parameters met the normality assumption, nonparametric statistics were used throughout this work. First, as no significant differences were observed between males and females' behaviors (Wilcoxon-Mann-Whitney (WMW) test) or in the behaviors of the 6 crab individuals of each control groups (Kruskal-Wallis test and Khi-square test), males and females, as well as individuals from different control groups, were pooled for subsequent analyses. Kruskal-Wallis test (KW test hereafter) was conducted to assess the presence of potential differences between the control and the 4 treatments in startle time, scototactic parameters, thigmotaxis, vigilance behaviors and the total time spent active. Khi-square test (Chi^2 hereafter) was conducted to assess the potential differences in crab's personality, i.e. the number of bold vs. shy individuals, in the number of individuals entering a black area first and touching a black wall first, for the 4 leachate treatments compared to the control. KW tests were conducted to assess the potential difference in the time spent active or inactive while walling in black or white areas. KW and WMW tests were conducted using the software RStudio (R 4.0.3).

Table 1. List of additives found in the pellets of different polymer depending on their function. Abbreviations means: tributyl Acetyl Citrate (ATBC), benzyl butyl phthalate (BBP), 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE153), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE154), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE183), butylated hydroxytoluene (BHT), bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), di-allyl phthalate (DAIP), phthalates dibutyl phthalate (DBP), bis-2-ethylhexyl adipate (DEHA), di(2-ethyhexyl)phthalate (DEHP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), diisodecyl phthalate (DIDP), diisoheptyl phthalate (DIHP), dimethyl phthalate (DMP), nonylphenol monoethoxylate (NP1EO), nonylphenol (NPs), tributyl phosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCPP).

Polymer type	**Additive function**	**Additives found in pellets**
PE	Plasticizers	ATBC, BBP, DAIP, DBP, DEHA, DEHP, DEP, DIBP, DIDP, DIHP, DMP
	Antioxidants	BPA, BHT, BPF, BPS, NP1EO, NPs
	Flame retardants	BDE153, BDE154, BDE 183, TBP, TCEP, TCPP, TDCPP
PA	Plasticizers	DBP, DEP, DIBP, DMP
BP	Plasticizers	DEHA, DIBP
	Antioxidants	NPs
PLA	Plasticizers	DEP, DIBP, DMP

3. Results

3.1. Pellets composition

The analysis of additives in PE pellets revealed the presence of various plasticizers, antioxidants and flame retardants. In contrast, PA and PLA pellets contained a smaller range of plasticizers and BP pellets were composed of two plasticizers along with one antioxidant (see results of additives analyzes in Table 1).

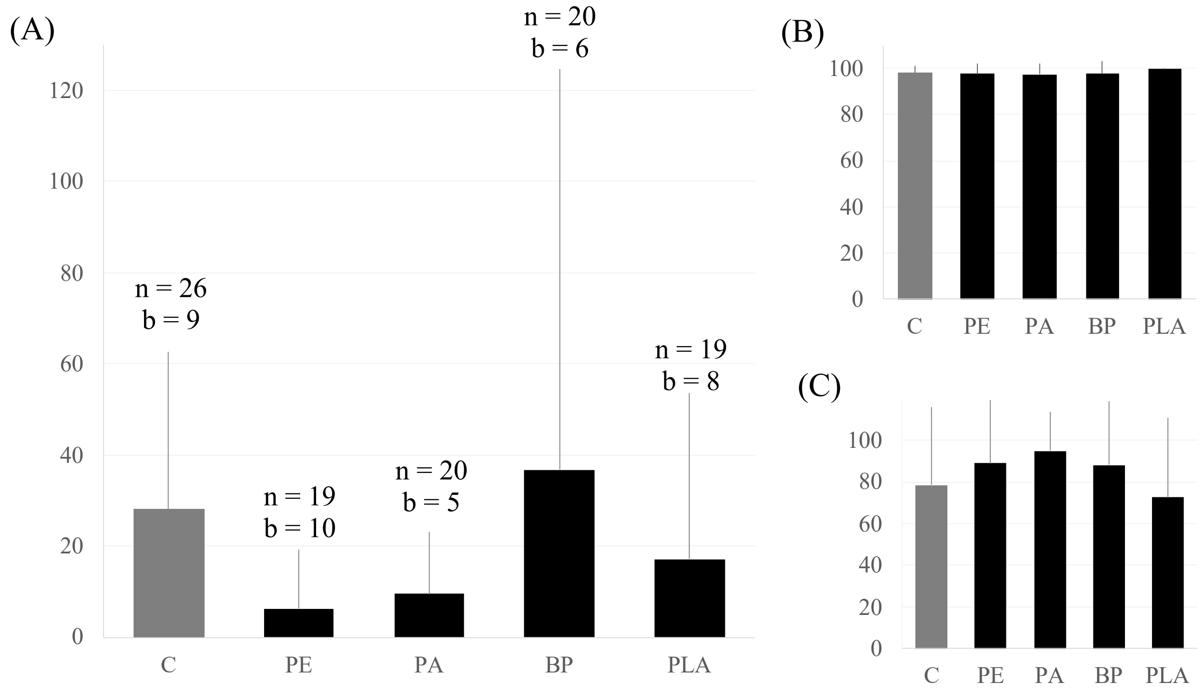


Figure 2. Anxiety behaviors of *Hemigrapsus sanguineus* after the 6 h-exposure to plastic pellet's leachate (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid; 24 h desorption; black bars). The figures represent the average time spent (A) in the central grey circle once the cage is lifted (s), i.e. startle time, (B) in contact with discontinuities once the central grey circle is left (%), i.e. thigmotaxis, and (C) with eyes directed toward the center of the arena when walling (%), i.e. vigilance, during the 10 min-record depending on the polymer type. “C” is the control group (grey bars). Errors bars are standard deviation, n is the number of analyzed individuals for each treatment (identical for each panel) and b is the number of bold individuals.

3.2. *Hemigrapsus sanguineus* anxiety behaviors

3.2.1. Startle time

Control crabs spent between 0 and 117 sec on the central grey circle once the acclimatization cage was lifted, with 37.5 % of individuals leaving the central grey circle in less than 1 sec (i.e. 9/24 bold individuals, Fig. 2A). The exposure to plastic leachates did not significantly impact either the startle time (KW test, Fig. 2A) or the number of bold individuals (Chi^2 test, Fig. 2A). The control group showed high interindividual variability in startle time (CV = 123 %) and the leachate exposure treatments exhibited CV values ranging from 144 to 248 % (Table 2).

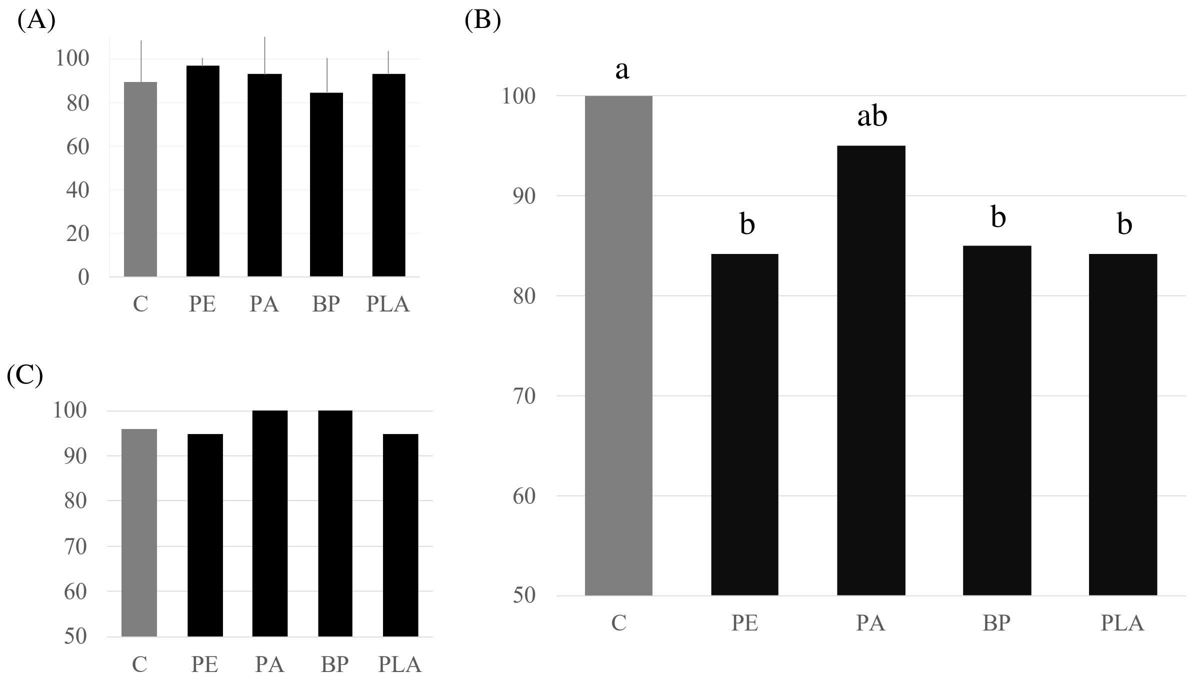


Figure 3. Scototactic behaviors of *Hemigrasus sanguineus* observed during the 10 min-record after a 6 h-exposure to the leachates of plastic pellets (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid; 24 h desorption; black bars). The figures represent (A) the average time spent in black areas (%), i.e. scototaxis, (B) the number of individuals observed to enter a black area first after leaving the central grey circle (%) and (C) the number of individuals observed to first touch a black wall (%). “C” is the control group (grey bars). Errors bars are standard deviation and letters above the bars represent significant differences between treatments (Chi^2 test).

3.2.2. Scototactic behaviors

H. sanguineus individuals from the control group spent more than 89 % (SD = 19.47 %) of their time in a black area (i.e. scototaxis, Fig. 3A). Once they left the central grey circle, 100 % of crabs in the control group entered a black area and 95.8 % touched a black wall first (Fig. 3B&C). While no significant effect was observed on the scototaxis (KW test, Fig. 3A) or on the color of the first wall touched (Chi^2 test, Fig. 3C) when crabs were exposed to plastic leachates, significantly less individuals touched a black area first when exposed to PE, BP and PLA leachates (respectively, 84.2, 85.0 and 84.2 %) compared to the control (Chi^2 test, Fig. 3B). The scototaxis CV of the control group was 22 % and it ranged from 4 to 19 % in the leachate treatments (Table 2).

Table 2. Variability in the anxiety behavior of *Hemigrapsus sanguineus* exposed to natural seawater (i.e. “C”) or solutions of plastic pellet leachates (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid) desorbed during 24 h assessed through the coefficient of variation (%) of the startle time (time spent in grey once the cage is lifted), the scototactic response (time spent in black areas once the central grey square is left), the thigmotactic response (time spent in contact with a discontinuity once the central grey square is left) and the vigilance response (time spent with eyes’ direction toward the center of the arena while walling or cornering). The record of behaviors lasted 10 minutes.

Treatment	Startle time	Scototaxis	Thigmotaxis	Vigilance
C	123	22	3	47
PE	203	4	4	34
PA	144	19	5	20
BP	248	18	6	35
PLA	214	11	0.31	53

3.2.3. Thigmotaxis

Individuals in the control group spent more than 98 % (SD = 2.74 %) of their time in contact with a discontinuity (i.e. thigmotaxis, Fig. 2B). No significant impact was observed when they were exposed to plastic leachates (KW test, Fig. 2B). The control group displayed low thigmotaxis CV (3 %) and the leachate exposure groups had CV values ranging from 0.31 to 6 % (Table 2).

3.2.4. Vigilance

In the control group, the time crabs spent with eyes toward the center of the arena was > 78 % (SD = 37.31, Fig. 2C). Exposure to plastic leachates did not yield significant differences in the vigilance behavior (KW test, Fig. 2C). The interindividual variability (CV) in vigilance was between 20 and 53 %, with the control group having a CV of 47 % (Table 2).

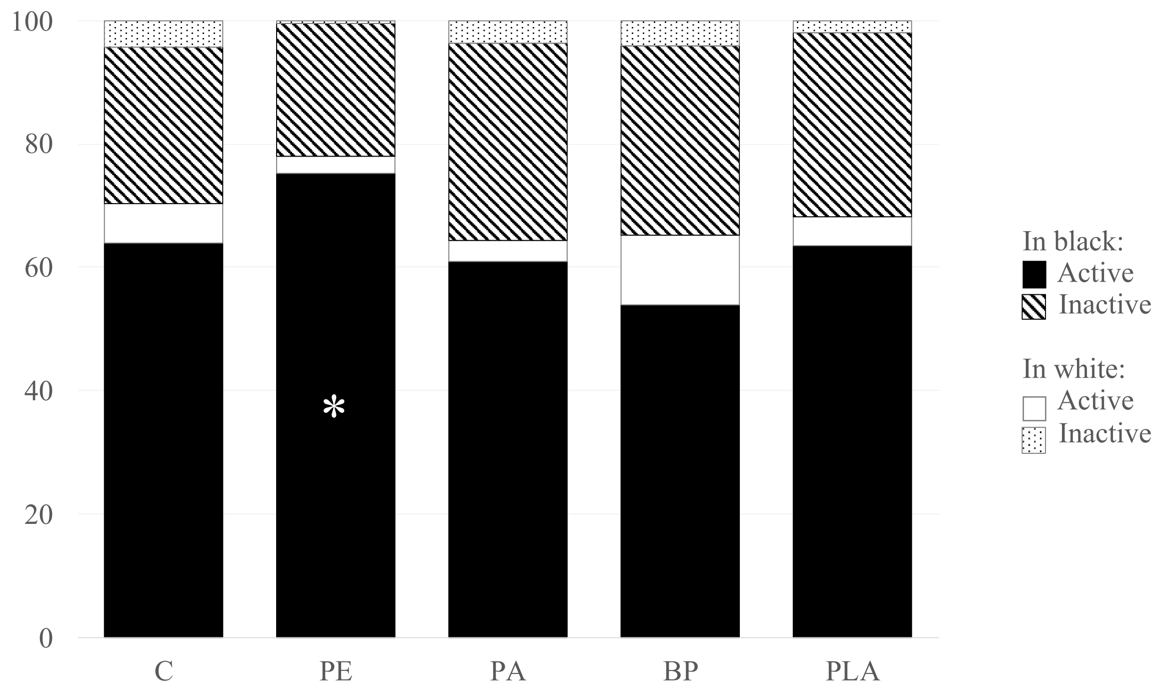


Figure 4. Percentage of the time *Hemigrapsus sanguineus* spent being either active or inactive in black or white areas, after 6 h-exposure to plastic pellets' leachates (24 h desorption); shown as a function of the polymer type (PE, polyethylene; PA, polyamide; BP, beached pellet; PLA, polylactic acid). “C” is the control treatment. Significant difference between treatments for each activity level between treatments is represented by a star (Kruskal-Wallis tests).

3.2.5. Activity level

In the control group, the total time spent active represented 70 % (SD = 19%, Fig. 4). When exposed to plastic leachates, the total time spent active ranged between 64 to 78 % with no significant differences with the control group (KW test, Fig. 4). Crabs were, however, significantly more active in black when exposed to PE leachates compared to other treatment (KW test, Fig. 4).

4. Discussion

4.1. Plastic leachates' impacts on crabs' behavior and contamination pathways

Our study corroborates previous results from a recent investigation into *H. sanguineus* anxiety behaviors (Delaeter et al., 2023). Our findings confirmed a range of behaviors exhibited by these crabs, including a short startle time, positive scototaxis, strong positive thigmotaxis and sharp vigilance behaviors; in addition to the highest and lowest inter-individual variations measured for scototaxis and thigmotaxis, respectively. Nevertheless, our assessment of the impact of plastic leachates on *H. sanguineus* anxiety behaviors stands apart from this previous study where surgical mask leachates did not induced alteration in crabs' behaviors. Indeed, we showed a significant increase in crabs' activity while walling in black following a PE exposure compared to the control group (Fig. 4). Dark areas are non-threatening environments (by comparison with white areas that are highly aversive environment), making activity level therein a proxy of performance (Maximino et al., 2010b). Indeed, when exposed to contaminants, organisms tend to increase their activity to cope with enhanced metabolic demands associated with the activation of detoxification pathways (White and Briffa, 2017). Consequently, PE exposure, triggering elevated activity level in dark areas compared to the control group, is likely to elicit an increase in metabolism processes.

Additionally, our study revealed a significant reduction in the number of crabs entering a black area following the removal of the acclimatization cage after an exposure to PE, BP and PLA leachates (Fig. 3b). *H. sanguineus*, exhibiting negative scototaxis, naturally seeks refuge in dark areas to avoid potential threats. Consequently, when facing a disturbance (such as the acclimatization cage removal), entering a white zone might constitute maladaptive behavior, rendering the crab more vulnerable to predators. Comparable maladaptive behaviors have been observed in other crab species: for example, hermit crabs exposed to plastic struggled to select suitable shells (Crump et al., 2023). Since entering a white or a black area require equivalent metabolic investment, plastic might impair behavior through an alternative mechanism. Behaviors are driven by physiological processes, i.e. the sensory system, the neurological functions, the endocrine system and the metabolism (Scott and Sloman, 2004). Contaminants can thus potentially alter organisms' behaviors through various pathways. As previously described, toxic chemicals can disrupt metabolic processes (White and Briffa, 2017), related to performances (Briffa and Sneddon, 2007); however contamination can also lead to 'info-disrupting', through the alteration of information gathering and decision-making processes (Lürling and Scheffer, 2007), i.e. cognition. Noticeably, additives found in the pellets used in the present experiments have been previously identified as endocrine-disrupting chemicals (e.g. BPA, DEHP or NPs; Zala and Penn, 2004) that can impact hormonal secretion (Sloman, 2007), metabolic processes and cognitive capabilities (Briffa et al., 2012; Briffa and Sneddon, 2007). Future investigations into oxidative stress markers may be needed to understand how plastic leachates disrupt physiological processes, resulting in modifications in individual behaviors.

4.2. Bioplastics: not such a good idea

Noticeably, crabs' behavior has been modified when exposed to PLA, a bio-based and biodegradable alternative to PE and PET (Ali et al., 2023). While bioplastics are often marketed as environmentally friendly solutions, they share similar issues with conventional fossil fuel plastics. Studies have demonstrated that they do not necessarily degrade faster in seawater (Cristina et al., 2022) and potentially generate more microplastics. PLA, for instance, generates a number of microparticles almost 3 times > to what is generates by fossil fuel plastics (Yang et al., 2022). Bioplastics can also adsorb higher contaminant levels than non-degradable plastics (Gong et al., 2019; Shi et al., 2023; Torres et al., 2021) and readily leach their additives (Quade et al., 2022), rendering them equally harmful to aquatic organisms as fossil fuel plastics. Bioplastics' impact on marine fauna can rival or surpass that of conventional plastics, negatively affecting diverse species. For example, PLA had stronger adverse effect on the oyster respiration rate compared to PE exposure (Green, 2016), showed detrimental impacts on phytoplankton assemblages unlike PS which showed no such effect (Yokota and Mehlrose, 2020), induced crab's maladaptive behaviors and increased activity level to disturbance whereas PA did not (this study). This stresses that, despite their marketed eco-friendliness, bioplastics may not be the solution to the plastic pollution issue, emphasizing the importance of including bioplastics in studies that investigate the impacts of plastics and their leachates on marine organisms.

4.3. Methodological aspects

Comparisons between our study and Delaeter et al. (2023) reveal differences in *H. sanguineus* behaviors. For example, we observed that the number of bold individuals (i.e. 61% vs. 37.5%, from Delaeter et al. (2023) and this study respectively) differed. In addition, differences occurred in the CV of scototaxis (i.e. 42% vs. 22%) and vigilance (184% vs. 47%). These variations can be attributed to methodological differences, such as the arena shape (square vs. circular), known to influence behaviors (Shimizu 2020). Noticeably, the circular arena design employed in our study prevents crabs from staying motionless in corners, potentially influencing their behavior. Furthermore, *H. sanguineus* being photophobic

(Spilmont et al., 2015), the use of indirect lightning possibly led to increase exploration, i.e. boldness, and activity. Mitigating visual disturbances during cage removal and experimentation may have further encouraged explorative behaviors. Finally, it is worth noting that the seasonal gap (spring vs. winter) between the two studies could also contribute to observed differences in crab behaviors.

Finally, despite its notably similar composition with PLA, it is noteworthy that PA pellets did not elicit any adverse effects on the investigated crab behaviors. Similarly, PA pellets were found to have no impact on the survival and reproduction of *Daphnia magna* (Khosrovyan and Kahru, 2022). Findings from another study showed that PA leachates did not affect zebrafish hatching, survival or body length (Zhang et al., 2022). Nevertheless, it is important to acknowledge that the composition of pellets, even within the same polymer type, can differ significantly based on their additives' composition, consequently leading to potential variations in their impacts. Furthermore, our analysis of pellet composition encompassed a spectrum of 57 additives and it is likely that untargeted chemicals can contribute to the observed behavioral disturbances during exposure to BP, PE and PLA. Notably, heavy metals, which can be found in the composition of plastic polymers, are recognized contributors to info-disruption (Boyd, 2010) and, as such, may play a role in behavioral modifications.

5. Conclusion

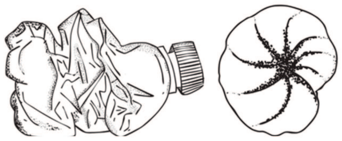
Changes in behavior is often the first response to anthropogenically disturbed environments (Mench, 1998; Wong and Candolin, 2015). Behavioral investigations enable us to assess the effects of contamination at several levels of organization, linking the individual to the ecosystem. In this study, we demonstrated a significant polymer dependent impact of plastic leachates on the behavioral response of *H. sanguineus* to a disturbance and the activity level. We suggested that those modifications in crabs' behaviors are related to impairments in their metabolic and cognitive capacities. Such alterations in organisms' behavior may enhance the vulnerability of crabs against predators modifying the prey-predator relationships, which may have far-reaching consequences through cascading direct and indirect effects at the community level, influencing ecosystem functioning. It is worth noting that *H. sanguineus* is an invasive species that exhibits high behavioral variability (Delaeter et al., 2023; this study), enabling it to cope with wide range of environmental conditions. The observed changes in *H. sanguineus* behaviors, which may jeopardize its survival, underscore the necessity to investigate the impact of plastic leachates on autochthone species, that typically exhibit lower tolerance to environmental changes.

Furthermore, we highlighted the potential of bio-based and biodegradable polymer to affect organism behavior in the same way as conventional polymers. Although often presented as the solution to plastic pollution, our results strongly question their role in mitigating the plastic's impact on marine environments, urging further investigation into the actual benefits of bioplastics within a plastic-saturated planet.

Acknowledgment

Alexandre Gravelle is acknowledged his contribution to the preliminary experiments during its internship. The ANSES is thanked for conducting the additives identification.

CHAPTER IV



POLYMER, TRAIT AND DOSE-DEPENDENCY OF THE IMPACT OF PLASTIC LEACHATES ON *HAYNESINA GERMANICA* MOTION BEHAVIOR.

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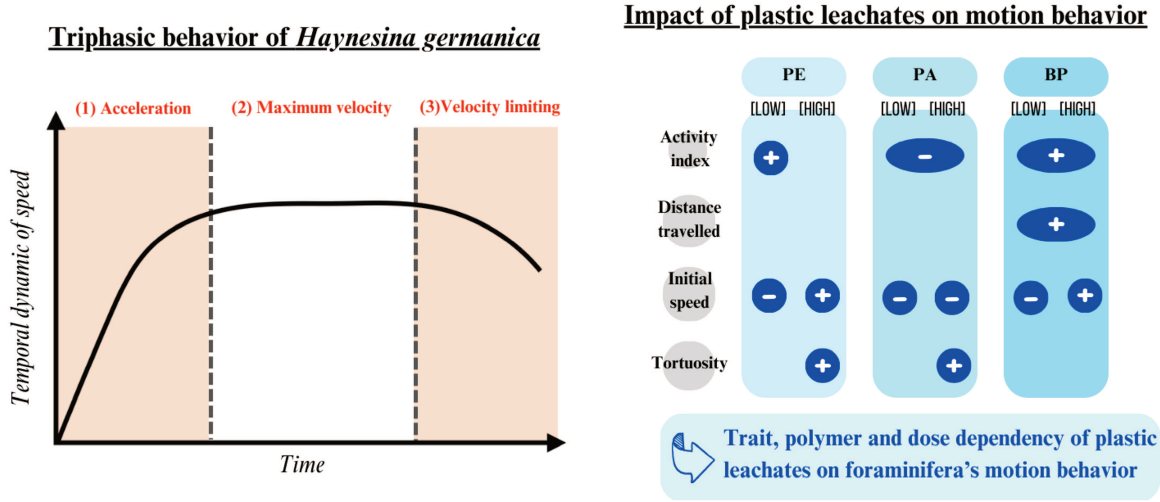
Graphical abstract



Abstract

Plastic pollution has become a pervasive threat to the marine environment in the Anthropocene era. Found from macro- to nanoscale, plastic items can leach a range of additives and persistent organic pollutant, adversely affecting organisms. While existing literature has demonstrated impacts at various organismal level, the impact of plastic leachate on foraminifera has been investigated only once, revealing no effect of polypropylene leachates on *Haynesina germanica* motion behavior and respiration rates. Toxicity of plastic leachates being highly dependent on the polymer type, we investigated the effects of plastic leachates from polyethylene (PE), polyamide (PE) and beached pellet (BP) at two concentrations on the motion behavior of *H. germanica*. Beyond conventional motion endpoints, we adapted the photosynthesis-irradiance model of Platt et al. (1980) in order to modelized the triphasic temporal dynamic of speed of *H. germanica*. Our findings underscored a trait, polymer and dose dependency in the impacts of plastic leachates. Low concentrations of PE induced an increase in the activity index and a decrease in the initial speed, whereas high concentrations led to an initial burst of activity and increased trajectory tortuosity. PA leachates significantly reduced the activity index and the initial speed along with an increase in trajectory tortuosity. Neither PE nor PA affected the total distance travelled by *H. germanica*. However, BP leachates increased both the activity index and distance traveled. Similar to PE, low and high concentrations of BP leachates induced a respective decrease and an increase in the initial speed. These behavioral modifications may reflect stress responses to contamination, physical and physiological impairments, posing a threat to foraminifera survival. Considering the role of foraminifera in bioturbation and bio-irrigation processes, such alterations may ultimately have impact on the functioning of intertidal mudflat ecosystems.

Keywords: plastic leachates, *Haynesina germanica*, motion behaviors, polymer-dependence

1. Introduction

Foraminifera, amoeboid protozoans characterized by a calcareous test and pseudopodia (i.e. arm-like extension of cytoplasm involved in feeding and motion; Murray, 2006), constitute a substantial (about 50%) portion of eukaryotic benthic biomass (Moodley et al., 2000). Inhabiting diverse environments, from the intertidal zone to the deep ocean (Goldstein, 1999), they play a crucial role as intermediaries between primary and secondary producers (Chronopoulou et al., 2019; Gooday et al., 1992; Nomaki et al., 2008; Wukovits et al., 2018). Beyond their central trophic functions, foraminifera contribute significantly to organic matter mineralization, nutrient cycles at the water-sediment interface and carbonate production (Langer, 2008). Furthermore, they contribute to the nitrogen cycle through nitrate accumulation and anaerobic respiration (Piña-Ochoa et al., 2010; Risgaard-Petersen et al., 2006).

Additionally, foraminifera exhibit the ability to move over relatively long distances (Seuront and Bouchet, 2015), a factor recently recognized as influencing bioturbation processes (Deldicq et al., 2020). Motion is fundamental for all organisms, integral to essential processes such as reproduction, feeding and survival, subsequently playing a pivotal role in shaping the structure and function of populations, communities and ecosystems (Hastings et al., 2011; Nathan et al., 2008). Foraminifera motion behavior actively contributes to sediment bioturbation and bio-irrigation processes, such as mixing enhancement (Deldicq et al., 2023, 2020), reduction of cohesiveness (Cedhagen et al., 2021), and facilitation of sediment resuspension in the water column (Orvain et al., 2004). They also create burrows, further increasing the fluxes of dissolved elements at the sediment-water interface (Hemleben and Kitazato, 1995). These processes, in turn, have direct repercussions on microbial communities (Mermillod-Blondin and Rosenberg, 2006; Piot et al., 2013), organic matter mineralization and nutrient cycles (Aller, 2014; Gilbertson et al., 2012), influencing the benthic-pelagic coupling and the overall ecosystem functioning (Danovaro et al., 2008; De Goeij et al., 2013). Any alteration of foraminiferal motion behavior may therefore lead to significant modifications in the structure and functioning of benthic ecosystems.

The role of motion behavior gains additional significance when considering species' ability to withstand environmental challenges induced by human activities (Sih et al., 2011). Plastic debris, one of the most widespread and conspicuous sources of pollution of the Anthropocene, heavily affect intertidal environments, which represents up to 90 % of litter on shorelines (Galgani et al., 2015). Found from macro- to nanoscale in the marine environments, plastic items can leach a range of additives and persistent organic pollutants with detrimental effects on organisms, affecting e.g. cellular activities, survival and also behavior; see Delaeter et al., (2022) for a review. Foraminifera, given their ecological characteristics (e.g. short life cycle, high abundance), have been extensively studied as bio-indicators of various pollutants, e.g. trace metals (Armynot du Châtelet et al., 2004), oils spills (Ernst et al., 2006), aquaculture pollution (Pawlowski et al., 2014; Vidović et al., 2014) or urban sewage (Burone et al., 2007). Nonetheless, to the best of our knowledge only four studies have specifically focused on the interplay between plastic and foraminifera through the assessment of the interaction with plastic particles (Birarda et al., 2021; Joppien et al., 2022a, 2022b) or the impact of plastic leachates (Langlet et al., 2020). Noticeably, only one study specifically focused on the impact of plastic on foraminifera movement behavior and showed that the exposure of *Haynesina germanica* to polypropylene (PP) leachates did not induce any change in its motion behavior nor respiration rate (Langlet et al., 2020).

Haynesina germanica, one of the most abundant species found in intertidal mudflats (Francescangeli et al., 2020), is likely to be exposed to substantial quantities of plastic debris, and this exposure is expected to increase in the future due to the ever-growing plastic pollution (Zalasiewicz et al., 2016). Recently, *H. germanica* has been described as a surficial biodiffusor (Deldicq et al., 2020), that plays a crucial role in sediment reworking due to its high activity level compared to other foraminiferal species (Deldicq et al., 2023, 2020). Despite the recent evidence of the lack of effect of PP leachates on the behavior and metabolism of *H. germanica* (Langlet et al., 2020), the impacts of plastics and their associated chemicals are fundamentally dependent on the polymer type and its chemical composition (Delaeter et al., 2022). In this context, this study aims to narrow the knowledge gaps in our understanding of the interplay between plastic pollution and benthic foraminifera through the assessment of possible impact of leachates from various polymers on the motion behavior of *H. germanica* given the importance of this species in the functioning of intertidal ecosystem. Polyethylene (PE), polyamide (PA) and beached pellets (essentially PE) have been chosen; PE being the most commonly produced (PlasticsEurope, 2021) and encountered polymer type in the environment (Erni-Cassola et al., 2019), PA being the forgotten polymer in the plastic literature especially compared to its presence in the environment (Delaeter et al., 2022) and BP are likely to accumulate substantial persistent organic pollutants on their surface (Fries and Zarfl, 2012).

2. Materials and methods

2.1. Organism' collection

Surface sediment was collected from the Bay of Authie mudflat, France (50°22'20.6"N, 1°35'45.5"E) in May 2022 by scraping the surface sediment using a spatula down to a depth of ca. 1 cm. This mudflat has specifically been chosen due to its relatively pristine properties and lack of anthropogenic forcing, as previously done in the context of foraminifera behavioral studies (Deldicq et al., 2023, 2021, 2020; Seuront and Bouchet, 2015). The sediment was stored in 100 mL polypropylene pots during transport and acclimatized overnighted in running natural seawater in an aquarium at field temperature (i.e. 14°C). The following morning, the sediment was sieved through a 125 µm mesh prior to inspection under binoculars to collect *Haynesina germanica* individuals. Individuals were then placed on markers drawn on a glass petri dish containing natural seawater, and a motility test was performed to select living individuals (i.e. those that moved from the original marker). For each treatment (control and plastic leachates; see below), thirty individuals were consistently selected prior to the start of the experiment. As preliminary experiments revealed significant differences in the behavioral properties when foraminifera were kept in an aquarium for several days (Delaeter, pers. obs.), fresh sediment was systematically collected the day before each experimental session (i.e. one per polymer type).

2.2. Leachate solutions preparation

The effects of leachates from three types of plastic were studied on the motion behavior of *Haynesina germanica*: virgin polyethylene pellets (PE; Materialix Ltd.), virgin polyamide pellets (PA; Akulon F136-C) and beached pellets (BP; collected from the Neufchâtel-Hardelot beach in France, 50°38'27.2"N, 1°34'37.4"E) which are typically made of PE (Zardi et al., accepted). Each experimental session consisted of one control solution (i.e. natural seawater) and two leachate solutions (i.e. low and high concentration) for each polymer type. These concentrations were respectively set as 10 and 50 g/L for cylindrical PE pellets (4.0 × 2.0 mm, diameter × heigh). To enable comparisons between treatments, the concentrations for the other pellet types were chosen based on their available exchange surface, i.e. 10 and 50 g/L of PE pellets corresponded to 11.5 and 57.4 g/L of cylindrical BP (3.5 × 3.1 mm) and 10.7 and 53.3 g/L of ellipsoidal PA (2.2 × 3 mm). For the sake of simplicity, these treatments were referred to as PE_{low} and PE_{high}, BP_{low} and BP_{high} , and PA_{low} and PA_{high} throughout the manuscript. Pellets were incubated in the dark in 100 mL of natural aerated seawater during 24h in temperature-controlled incubators set to 15°C, and aerated using an air pump connected to a glass Pasteur pipette. A 100 mL seawater control solution (without pellets) was incubated under the same conditions. After the 24-hour incubation, the pellets were separated from the water via sieving (mesh size 2 mm), and the solutions were promptly used for the experimental session.

2.3. Behavioral assay

Behavioral experiments were conducted in glass Petri dishes (inner diameter 10 cm) filled with 80 mL of either control seawater or leachate solution, where 30 motile *Haynesina germanica* individuals were placed on the bottom using a fine brush. Petri dishes were subsequently placed in temperature-controlled incubators at a constant temperature (15°C). A digital camera (Nikon V1 with a Nikkor 10-30mm lens) was placed at 20 cm above each Petri dish, and one image was taken every 10 minutes for 20 h (i.e. $N = 120$ images) to assess the motion behavior of the foraminifera.

2.4. Behavioral analysis

The movement behavior of *H. germanica* was subsequently further quantified through their level of activity (A_i; %) estimated as the percentage of time allocated to movement (t_{active}) over the experiment duration (t_{total}, i.e. 20 h) as:

$$Ai \; = 100 \times \frac{t_{active}}{t_{total}} \tag{1}$$

The coordinates of the positions of each foraminifera were subsequently extracted from the pictures using the plugin Manual Tracking on ImageJ (ImageJ2 2.9.0/1.53t; Schindelin et al., 2012). The distance, D_t (mm), moved by an individual between two successive pictures was obtained as:

$$D_t = \sqrt{(x_t - x_{t+1})^2 + (y_t - y_{t+1})^2} \tag{2}$$

where (x_t, y_t) and (x_{t+1}, y_{t+1}) are the coordinates of a specimen at time t and $t+1$. The total distance (D_{20}) moved throughout the experiment was subsequently obtained by adding the distances moved between each picture (D_t, i.e. $D_{20} = \sum_{t=0}^{t=120} D_t$). The instantaneous movement speed of a specimen *i* (v_i; mm h^{-1}) was estimated for each *H. germanica* individual as $v_i = D_t \times f$, where f is the sampling rate of the camera (i.e. 1 image per 10 min converted in hours). The speed during the initial 10 min step (v_1) was isolated as a variable and the averaged instantaneous speed (v_t) was subsequently calculated over the 30 experimental individuals (i.e. $v_t = \frac{\sum_{i=1}^{i=30} v_i}{30}$) to assess the temporal dynamic of instantaneous speed. Given the shape of the distribution (see Fig. 3 hereafter) and by analogy with the photosynthesis-irradiance curves, the non-linear temporal dynamics of the instantaneous speed of movement was modelled following Platt et al. (1980) as:

$$V_t = V_{max}\left(1 - e^{-\frac{\alpha t}{V_{max}}}\right)e^{-\frac{\beta t}{V_{max}}} \tag{3}$$

where V_{max} is the maximal speed (mm h^{-1}), α the initial slope (mm h^{-2}), β a velocity limiting fitting parameter characterizing the diminution of V_t with time once V_{max} is reached (mm h^{-2}), and t the time (h). The function was fitted using RStudio (RStudio 1.3.1093).

Finally, the tortuosity of trajectories was calculated using the Net-to-Gross Displacement Ratio (NGDR), i.e. the dimensionless ratio between the net distance D_n (distance between the starting and final coordinates) and the total distance D_{20} as:

$$NGDR = \frac{D_n}{D_{20}} \tag{4}$$

2.5. Additives' composition assessment

The identification of the additives content of the plastic pellets was carried out using a CDS Pyroprobe 6150 pyrolyzer (CDS Analytical) in association with a GC-HRMS instrument (GC Trace 1310-MS Orbitrap Q Exactive, Thermo Fisher Scientific). Thermal desorption was performed (350 °C) to remove the potential additives from the samples. The samples were then separated using a Restek Rxi-5-MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with a cross-linked poly 5 % diphenyl-95 % dimethylsiloxane stationary phase (slip ratio: 1:5) and the acquisition was conducted on full-scan (FS) mode (m/z = 30.00000–600.00000). The resulting chromatograms were analyzed using Xcalibur and TraceFinder software for the identification of organic plastic additives among a selection of 57 additives (i.e. plasticizers, flame retardant, antioxidants and UVs stabilizers; see Supplementary material 1 for details). The subsequent identification of the additives was based on their retention times, m/z values, and specific ions, which were compared with the chromatograms obtained from standard solutions of each additive.

Table 1. List of additives found in the pellets of different polymer depending on their function. Abbreviations means: tributyl Acetyl Citrate (ATBC), benzyl butyl phthalate (BBP), 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE153), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE154), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE183), butylated hydroxytoluene (BHT), bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), di-allyl phthalate (DAIP), phthalates dibutyl phthalate (DBP), bis-2-ethylhexyl adipate (DEHA), di(2-ethyhexyl)phthalate (DEHP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), diisodecyl phthalate (DIDP), diisoheptyl phthalate (DIHP), dimethyl phthalate (DMP), nonylphenol monoethoxylate (NP1EO), nonylphenol (NPs), tributyl phosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCPP).

Polymer type	**Additive function**	**Additives found in pellets**
PE	Plasticizers	ATBC, BBP, DAIP, DBP, DEHA, DEHP, DEP, DIBP, DIDP, DIHP, DMP
	Antioxidants	BPA, BHT, BPF, BPS, NP1EO, NPs
	Flame retardants	BDE153, BDE154, BDE 183, TBP, TCEP, TCPP, TDCPP
PA	Plasticizers	DBP, DEP, DIBP, DMP
BP	Plasticizers	DEHA, DIBP
	Antioxidants	NPs

2.6. Statistical analyses

Statistical analyses were conducted using the software RStudio (RStudio 1.3.1093). Since none of the parameters met the normality assumption (Shapiro-Wilk test), nonparametric statistical tests were used. For each session of the experiment, differences in total distance moved (D_{20}), NGDR and activity index (Ai) between the control group and the treatments (i.e., low concentration and high concentration) were analyzed using the Kruskal-Wallis test, followed by Dunn tests when significant differences were identified.

3. Results

3.1. Composition of plastic pellets

The additives assessment revealed that PE pellets were composed of 26 additives, including 13 plasticizers, 6 antioxydants and 7 flame retardants. In sharp contrast, PA and BP pellets contained only 4 additives (4 plasticizers) and 3 additives (i.e. 2 plasticizers and 1 antioxydants), respectively; see Table 1 and Supplementary Materials 3 for more details.

3.2. Behavioral assays

Off the 270 individuals considered in the present work, only 3 individuals did not move (i.e. 1.1%) and 1 individual was discarded as it climbed the walls of the Petri dishes before the end of the behavioral experiment. This resulted in 28 individuals in the BP_{high} treatment and 29 individuals in the $BP_{control}$ and PE_{high} treatments. The other treatments (i.e. BP_{low}, $PE_{control}$, PE_{low}, $PA_{control}$, PA_{low} and PA_{high} treatments) were consistently based on 30 individuals.

Table 2. Level of activity of foraminifera exposed to virgin polyethylene (PE), virgin polyamide (PA) and beached pellets (BP) leachates assessed through the activity index. The mean (± standard deviation) and the minimal and maximal value are given for each treatment; low and high concentrations and their control (C). The significant differences between the leachate treatments and their respective control are represented by an asterisk (*).

	PE			PA			BP		
	C	Low	High	C	Low	High	C	Low	High
Mean (± SD) (%)	99.6 (± 0.8)	97.1 (± 3.5)*	97.7 (± 6.7)	96.7 (± 4.2)	93.7 (± 5.8)*	92.5 (± 6.8)*	92.4 (± 10.6)	98.2 (± 2.3)*	97.8 (± 2.8)*
[Min – Max] (%)	96.3 – 100	83.2 – 100	64.7 – 100	81.5 – 100	70.6 – 99.1	63.0 – 99.1	42.0 – 99.1	87.4 – 100	86.6 – 100

3.2.1. PE behavioral assay

The activity index A_i was consistently greater than 97% for each treatment (Table 2). Although significatively less time was allocated to movement under conditions of low leachate concentration, PE_{low} ($p < 0.05$; Table 2), no significant differences between treatments were observed in the total distance moved (Fig. 1).

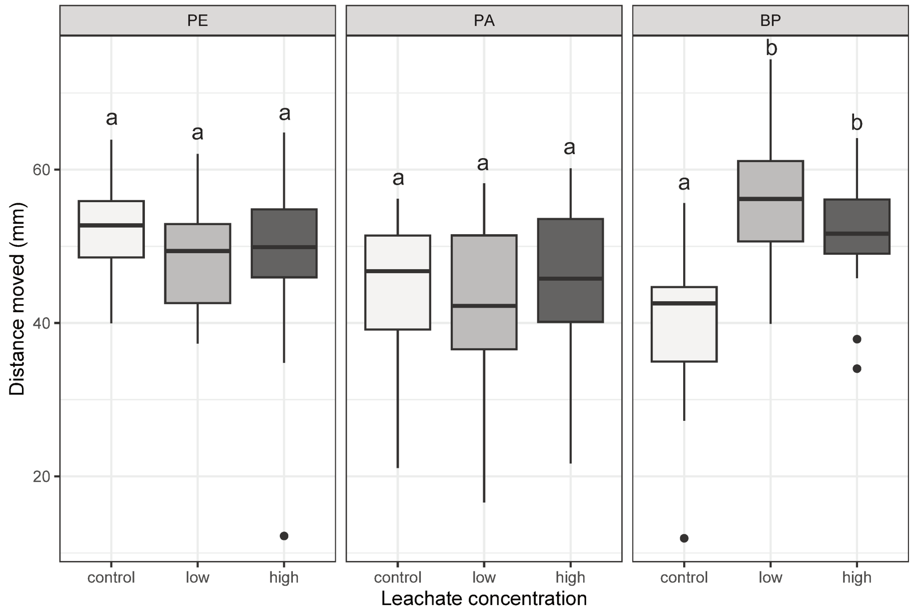


Figure 1. Boxplot of the total distance moved (mm) by *Haynesina germanica* during 20h-exposure to a low or high concentration of plastic pellet leachates from different polymer type. BP is beached pellets, PA is virgin polyamide pellets, PE is virgin polyethylene pellets. Potential significant differences are represented by letters above the boxes, different letters mean significant differences, each experimental session being analyzed independently from one another.

The distance travelled during the first 10 minutes significantly differ between all the PE treatments ($p < 0.05$; Fig. 2). Noticeably, this distance was more than one order of magnitude larger in PE_{high} compared to the control (Fig. 2). The temporal dynamic of instantaneous speed V_t was relatively consistent between $PE_{control}$, PE_{low} and PE_{high} (Fig. 3). We nevertheless observed slightly slower V_{max} values for both PE_{low} and PE_{high} compared to the control, together with a slight increase in the velocity limiting parameter β (Fig. 3, Table 3). A shallower slope

α was, however, observed for PE_{low}. Trajectories were significantly more complex when foraminifera were exposed to high leachate concentration, with a significant decrease in the NGDR value in the PE_{high} treatment (Fig. 4).

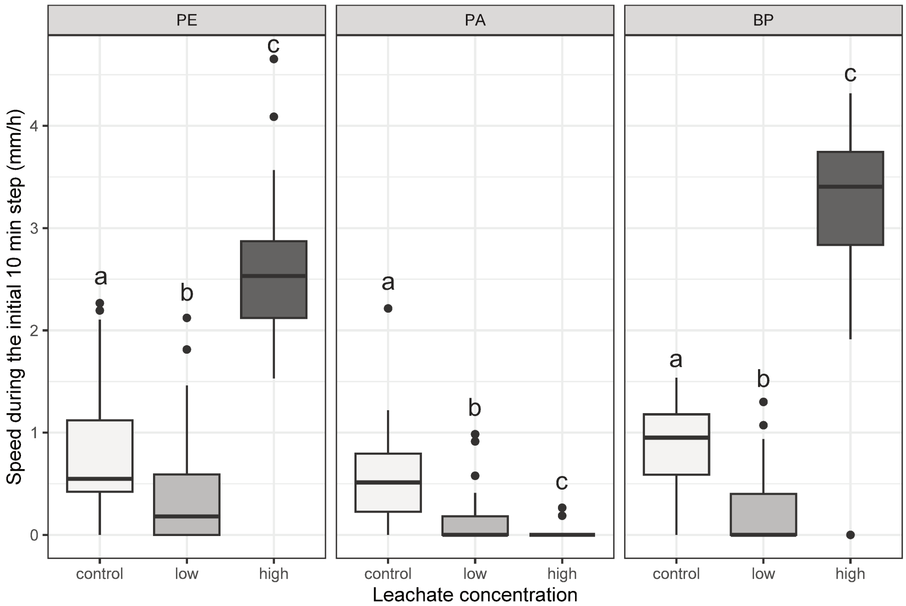


Figure 2. Speed (mm/h) of *Haynesina germanica* during the initial 10 minutes step when exposed to virgin polyethylene (PE), virgin polyamide (PA) and beached pellets (BP) leachates. Potential significant differences are represented by letters above the boxes, different letters mean significant differences, each experimental session being analyzed independently from one another.

Table 3. Parameters of the Pratt et al. (1980) equation (α, *Vmax* and β) and the coefficient of determination of the modelized temporal dynamic of speed of *Haynesina germanica* exposed to beached pellet (BP), polyamide (PA) and polyethylene (PE) leachates at low and high concentrations and their respective control (C). Units are mm/h for α and β, and mm/h^2 for *Vmax*.

	PE			**PA**			**BP**		
	C	**Low**	**High**	**C**	**Low**	**High**	**C**	**Low**	**High**
$\boldsymbol{\alpha}$	0,36	0,21	0,28	0,14	0,14	0,11	0,10	0,22	0,22
Vmax	2,82	2,70	2,76	2,76	2,40	3,84	2,76	3,06	3,18
$\boldsymbol{\beta}$	$6.1\ 10^{-4}$	$1.0\ 10^{-3}$	$1.4\ 10^{-3}$	$2.6\ 10^{-3}$	$4.3\ 10^{-5}$	$1.1\ 10^{-2}$	$4.3\ 10^{-3}$	$4.3\ 10^{-4}$	$3.6\ 10^{-3}$
R^2	0.49	0.61	0.63	0.72	0.77	0.87	0.84	0.82	0.77

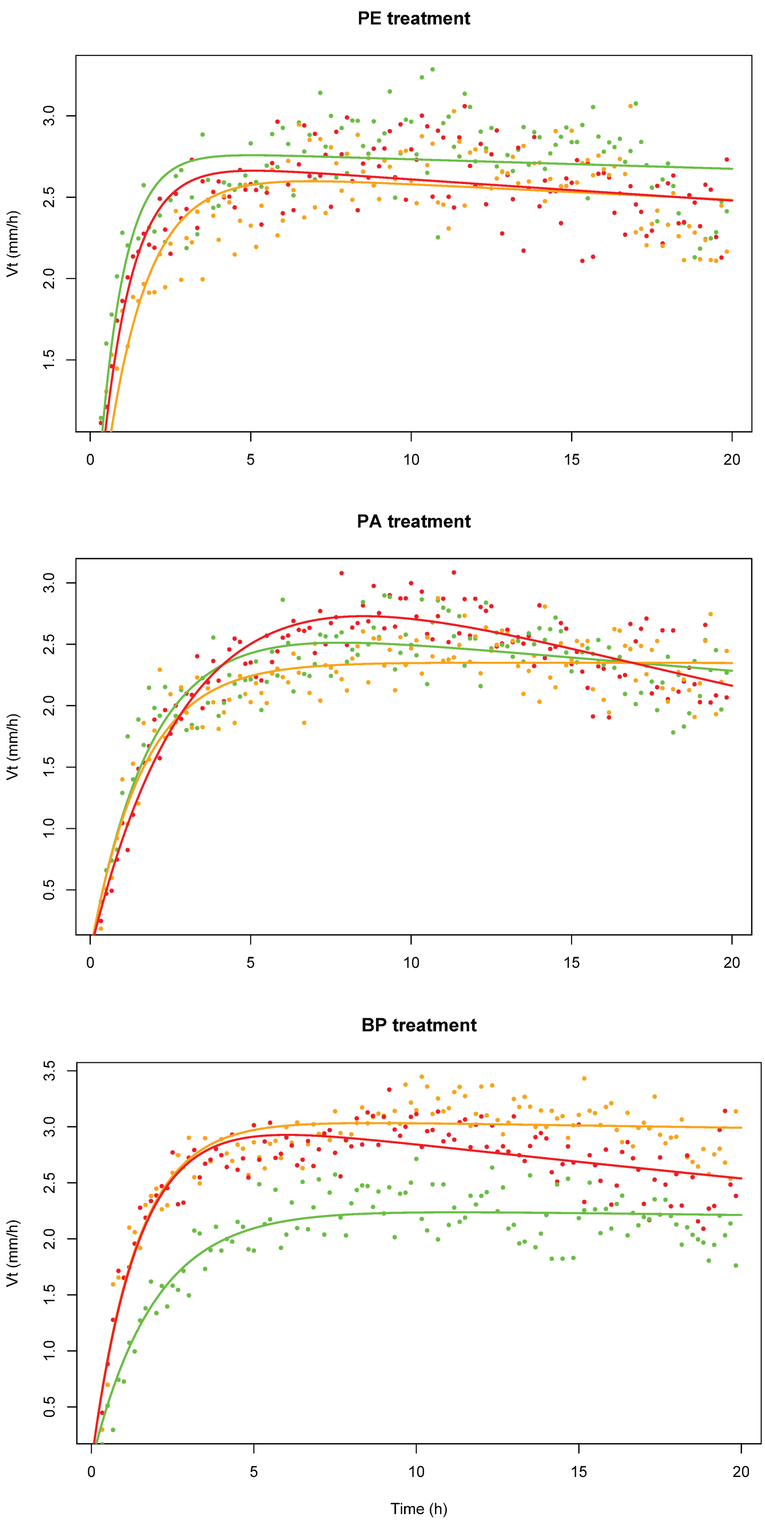


Figure 3. Temporal dynamic of the mean speed of *Haynesina germanica* when exposed to virgin polyethylene (PE), virgin polyamide (PA) and beached pellets (BP) leachates. Each graph represents the experimental distribution of the mean speed (i.e. v_t calculated at each time step, i.e. 10 min, on the 30 individuals; dots) and the modelized distribution using the Pratt et al. (1980) photosynthesis – irradiance equation (i.e. V_t; line) of the control group (green), the low concentration (BP_{low}, PA_{low} and PE_{low}; orange) and the high concentration (BP_{high}, PA_{high} and PE_{high}; red).

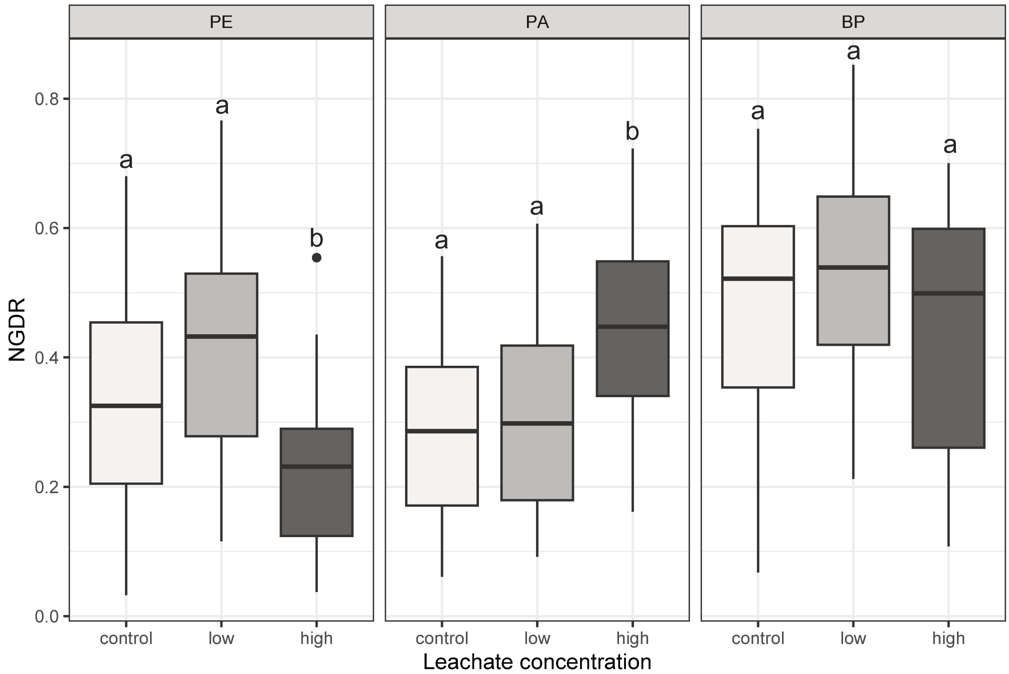


Figure 4. Boxplots representing the NGDR of the trajectories of *Haynesina germanica* during a 20h-exposure to a low (10) or high (50) concentration of plastic pellet leachates from different polymer types. PE is virgin polyethylene, PA is virgin polyamide, BP is beached pellet. Potential significant differences are represented by letters above the boxes, different letters mean significant differences, each experimental session being analyzed independently from one another

3.2.2. PA behavioral assay

Individuals were significantly less active (Dunn test, $p < 0.05$) when exposed to PA leachates (PA_{low}: 93.7 ± 5.8 %, PA_{high}: 92.5 ± 6.8 %) than in control seawater (96.7 ± 4.2 %; Table 2). No significant differences were, however, observed in the distance moved (Fig. 1), suggesting that individuals move less but over larger distance when they do.

In contrary to what has been observed in the PE behavioral assay, the distance travelled during the first 10 minutes in the treatment PA_{high} were significantly smaller than in PA_{low} and the corresponding control experiment ($p < 0.05$; Fig. 2). The temporal dynamic of the instantaneous speed V_t essentially differed in the velocity limiting parameter β observed in the PA_{low} and PA_{high} treatments which were respectively 60-fold lower and 4-fold higher than the value characterizing the control group (Fig. 3, Table 3).

Finally, the tortuosity of the trajectories was significantly lower when foraminifera were exposed to high leachate concentrations (PA_{high}) compared to the control (0.44 ± 0.16 and 0.28 ± 0.15 respectively; Dunn test, $p < 0.05$; Fig. 4).

3.2.3. BP behavioral assay

When exposed to BP leachates, *H. germanica* allocated significantly more time to movement than in control seawater (Dunn test, $p < 0.05$; Table 2), resulting in a distance moved significantly higher in BP_{low} and BP_{high} treatments (D_{20} = 56 ± 8 and 52 ± 7 mm, respectively) compared to the control (D_{20} = 40 ± 9 mm; Fig. 1). Similarly to PE treatments, the distance travelled during the first 10 minutes in the treatment BP_{high} were significantly higher than in BP_{low} and $BP_{control}$ experiment ($p < 0.05$; Fig. 2). In sharp contrast with the observations conducted in the PE and PA assays, the temporal dynamic of the instantaneous speed in the BP_{low} and BP_{high} treatments was noticeably characterized by an increase in velocity α (i.e. α = 0.22 mm h^{-2}; Table 3) that was more than twice as high as the value obtained when *H. germanica* were exposed to control seawater (Fig. 3). Under conditions of high leachate concentrations (BP_{high}), V_t was characterized by a drastic decrease (i.e. one order of magnitude) in the velocity limiting parameter β, compared to BP_{low} and $BP_{control}$ (Table 3, Fig. 3). Finally, no significant differences were observed in the tortuosity of *H. germanica* trajectories (Fig. 4).

4. Discussion

The assessment of the role of plastic leachates in the biology and the ecology of marine invertebrates is an emerging topic in the plastic pollution literature, which has been exponentially growing over the past decade; see Delaeter et al. (2022) for a review. In this context, it is noticeable that despite the increasing use of foraminifera as bio-indicators in relation to anthropic pollution (Armynot du Châtelet et al., 2004; Burone et al., 2007; Ernst et al., 2006; Pawlowski et al., 2014; Vidović et al., 2014), and growing evidence of the incorporation of plastic particles in foraminifera tests (Birarda et al., 2021; Joppien et al., 2022a; Tsuchiya and Nomaki, 2019), there is a critical lack of knowledge on the influence plastics may have on these organisms. To the best of our knowledge, only a handful of papers delt with the effect of plastic particles (Birarda et al., 2021; Ciacci et al., 2019; Joppien et al., 2022b) on foraminifera; see Bouchet et al. (2023) for a review. Specifically, the only work that investigated *sensu strictissimo* the impact of an exposure of a foraminifera to a leachate solution did not find any impact on the respiration rate nor the motion behavior of *Haynesina germanica* exposed to leachate from virgin polypropylene pellets, even at very high pellet concentrations (i.e. 200 mL of pellets per liter; Langlet et al., 2020). These results consistently point towards a relative lack of detrimental effects of plastic leachates on foraminifera. It is noticeable, however, that previous research in plastic leachates has shown that their impacts are polymer-dependent (Bejgarn et al., 2015; Capolupo et al., 2021, 2020; Delaeter et al., 2023; Seuront et al., 2021). This observation stresses a critical need in conducting multi-polymer studies—as previously suggested by Langlet et al. (2020)—to draw reliable and ecologically meaningful conclusions about the potential resistance of foraminifera, and *H. germanica* in particular, to the presence of plastic leachates in their environment.

In this context, we investigated the impact of leachates from different polymer types, i.e. PE, PA and BP (specifically selected for their relative abundance in environment and potential pollutant content) and, in sharp contrast with the available literature, we observed significant effects on various aspects of the motion behavior of *H. germanica*. Based on our unique adaptation of photosynthesis-irradiance model of Platt et al. (1980) to the temporal dynamics of velocity fluctuations, we demonstrated that *H. germanica* exhibited a clear triphasic behavior in the temporal dynamics of its velocity fluctuations, which were characterized by (i) a rapid phase of acceleration, (ii) a maximum velocity and (iii) a subsequent slow decay in the observed velocity. While this triphasic behavior was consistent irrespective of the polymer type and the related additive composition (Fig. 3), we found that the behavioral response of *H. germanica* was clearly polymer-dependent (results are summarized in Table 4).

Table 4. Summary of the significant differences observed in the motion behavior of *Haynesina germanica* exposed to the leachates of polyethylene (PE), polyamide (PA) and beached pellets (BP).

	PE		**PA**		**BP**	
Trait	low	high	low	high	low	high
Ai	↘		↘	↘	↗	↗
D_{20}					↗	↗
Initial speed	↘	↗	↘	↘	↘	↗
NGDR		↘		↗		

Indeed, PE exposure led to a reduction in the time allocated to motion under low concentration, while an increase in tortuosity and velocity during the initial 10-minute step was observed. Furthermore, general activity (i.e. *Ai*, D_{20}, initial 10 min speed and temporal dynamic of speed) increased when foraminifera were exposed to BP leachates. In contrast, exposure to PA leachates resulted in a decrease in the activity index and tortuosity. These observed disparities in the impacts on motion behaviors can be attributed to the plastic composition in additives, which varies depending on the polymer type, intended purpose of the final product and even manufacturers 'recipe', rendering it highly variable. Chemical analysis of pellets confirmed this significant variability and revealed a markedly different composition between the tested polymers. However, BP seemed more toxic than PE and PA, but surprisingly contained significatively fewer additives than PE. Additionally, while PE and PA are virgin pellets releasing primary leachates, BP have been aged and weathered in the environment and should potentially release primary and secondary leachates, explaining its toxicity. These results are consistent with recent results that showed that BP leachate inhibit the ability of the blue mussel *Mytilus edulis* to form aggregates (Zardi et al., accepted). Although the number of additives found on the pellets is not an indicator of the toxicity of leachates, it is worth noting that we only searched for 57 additives. Noticeably, potential components of secondary leachates recognized as toxic, such as heavy metals, PAH or PCB (Kedzierski et al., 2018; Ogata et al., 2009; Weis, 2019), were not targeted here and might have contributed to the observed behavioral disturbances.

Hyperactivity was observed under condition of exposure to high concentrations of both BP and PE leachates and could be associated to a flight response. The burst of activity observed during the first 10 minutes of these experiment is consistent with the startle response typically observed in anxiety-related studies in response to an undesirable (or stressful) environment, as previously observed in a wide range of organisms (Cribb and Seuront, 2016; Maximino et al., 2010a). Nevertheless, behaviors are driven by physiological processes and morphological abilities, and the additional alteration observed in motion behaviors must indicate further underlying damages beyond a simple flight response.

Metabolism is closely related to organisms' behavior and is acknowledged to be sensitive to plastic contamination (Capolupo et al., 2021; Green et al., 2016). In this work, BP induced a general increase in the temporal dynamic of speed, corroborated by the significant increase in the distance travelled. Furthermore, the increased activity level associated to the non-modified distance travelled under PA leachates indicates the adoption of a more erratic behavior. Altogether, the increase in general activity and distance travelled, the more erratic behaviors and the initial burst of activity are consistent with responses typical of anxiety behaviors and further correlate an increase in the organism's stress level (Brodin et al., 2014; Maximino et al., 2010a; Réale et al., 2007). The individuals' activity is described in anxiety-behavior studies as a trait that connects metabolism and behavior (Careau et al., 2008). This link is also relevant in a foraminiferal context. For example, Deldicq et al. (2021) showed that the impact of temperature on the metabolism of *H. germanica*, up to 30°C exposure, is correlated to its locomotor activity. In this study, although we did not specifically investigate the effect of plastic leachates on the metabolism, we observed modification in motion behaviors that could be attributed to a higher metabolic demand required for activating detoxification processes in cells (White and Briffa, 2017), corroborating the stress response hypothesis

In addition to polymer-dependency, we also observed dose-dependency. While stronger effects were observed under high leachate concentration, we also noticed the opposite impacts in motion behaviors between low and high concentration. Similar opposite dose-dependency effects have been documented in the literature (Trailović et al., 2017; Wallace et al., 2007). For example, diazinon had contrary effects on the motor activity of the rat ileum, increasing contraction at low dose and decreasing it at high concentration. The authors hypothesized that at high dose, diazinon had a blocking effect on the receptors they are binding at low concentrations by binding additional accessory site (Trailović et al., 2017). Components of plastic leachates may potentially induce similar types of neurological effects, explaining the differences observed in the initial 10-minutes speed between low and high concentrations of PE and BP treatments and further supporting the physiological damages hypothesis.

The observed modification in motion behaviors can also be induced by a locomotor deficit. Noticeably, previous studies have shown that exposure to pollutants can adversely affect the pseudopodal activity of foraminifera (Denoyelle et al., 2012; Le Cadre and Debenay, 2006; Nigam et al., 2009; Saraswat et al., 2004). Pseudopods are cytoplasmic extensions that branched to form a network (Bowser and Travis, 2002) allowing the locomotion of foraminifera through a “grip-and-tug” mechanism (Murray, 2006). They respond to environmental stimuli (Travis et al., 2002) through modifications in cytoplasm (Nigam et al., 2009; Saraswat et al., 2004) and/or metabolic rates (Cedhagen and Frimanson, 2002). For instance, exposure to heavy metals such as Hg and Cd has been shown to cause a decrease in pseudopodal activity in *Rosalina leei* (Nigam et al., 2009; Saraswat et al., 2004) and *Ammonia tepida* (Denoyelle et al., 2012). Pseudopodal activity even ceased when *A. tepida* and *Ammonia beccarii* were exposed to Cu (Le Cadre and Debenay, 2006). The decrease in *H. germanica* activity index observed in the PE_{low} and both PA treatments, may be related to the impact of leachates on pseudopodal activity. Noticeably, alterations in pseudopodal activity can have significant consequences on individual survival and ecosystem functioning. Indeed, pseudopods play a crucial role in many aspects of foraminifera biology and ecology, including respiration, attachment or movement, prey capture, test construction and reproduction (Goldstein, 1999; Murray, 2006 and references therein). Additionally, foraminifera, through their pseudopodal activity and associated motion behavior, are involved in bioturbation and bio-irrigation processes (Deldicq et al., 2023, 2020). Altogether, decrease in pseudopodal activity and increase of stress is likely to affect individual fitness and ultimately environmental processes.

In addition to a decrease in motion activity, our study showed significant modifications in behavioral complexity. While no significant impact was observed under BP leachate exposure, the PE treatments led to an increase in the tortuosity, i.e. a more intensive behavior. Conversely, the PA treatments resulted in opposite effects leading to a more extensive behavior. The tortuosity of trajectories is closely related to the foraging strategy adopted by organisms (de Jager et al., 2014; Reynolds, 2018; Viswanathan et al., 1999). Trajectories can be categorized as either intensive or extensive, depending on the adopted strategy (Reynolds, 2015, 2018; Sims, 2015). Previous study has shown that foraminifera are able to detect the presence of food and adjusting the tortuosity of their trajectories accordingly (Deldicq, 2021). In particular, they have demonstrated that *H. germanica*, which feed on patchily distributed diatoms, exhibit a relatively extensive motion behavior with long displacements. Our investigation of the impact of leachates on the NGDR confirmed a polymer dependency and revealed a dose dependency as impacts are observed at the highest concentration. Consequently, modifications in trajectory complexity, and underlying foraging strategy, could potentially result in reduced efficiency in the search for food, ultimately impacting the organism survival and having drastic implications in bioturbation and bio-irrigation processes.

5. Conclusion

This study shows, for the first time, a significant impact of plastic leachates on foraminifera. We observed significant modifications in the motion behavior of the benthic foraminifera *Haynesina germanica*, demonstrating a trait, dose and polymer dependency of these impacts. We suggest that the observed alterations are consequences for underlying damages in physiological processes and/or locomotor abilities. Considering the pivotal role of foraminifera activity in bioturbation and bio-irrigation of the sediment, the alteration of motion behaviors may not only impact individual survival but also affect the ecosystem functioning. In this context, it is noteworthy that Langlet et al. (2020) investigated the impact of plastic leachates on foraminifera collected from the highly polluted (in particular with heavy metals) harbor of Boulogne-sur-Mer, whereas our samples were obtained from the less polluted Baie d'Authie estuary. Considering that sudden exposure to pollutants has been shown to cause more impairments in foraminiferal growth than gradual exposure (Nigam et al., 2009), the lack of impact from PP leachate exposure on *H. germanica* motion behavior may be attributed to the relatively low toxicity of PP leachate or the potential development of resistance in individuals already inhabiting a highly polluted area. Further investigations are necessary to determine the resistance capacity of different populations to plastic pollution, which may vary depending on the pollution levels in their respective habitats.

Acknowledgment

The ANSES is thanked for conducting the additives identification.

Supplementary Materials 3

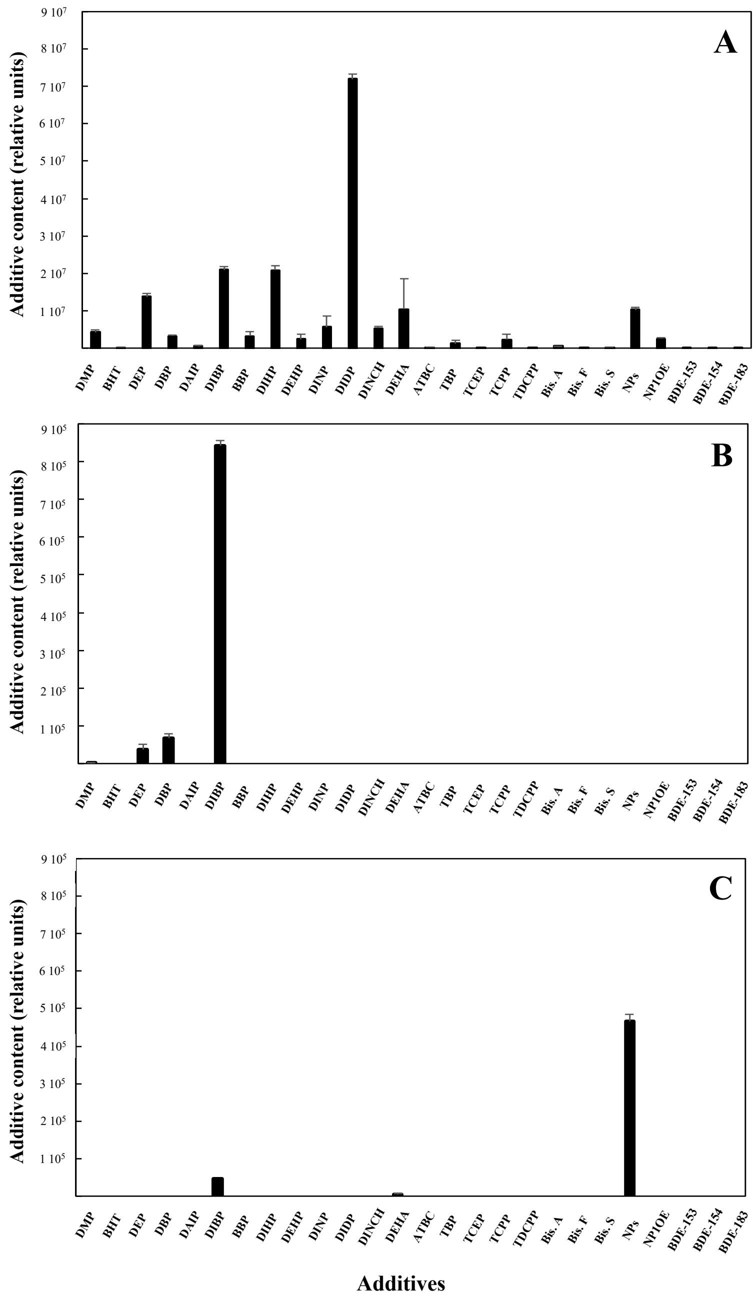
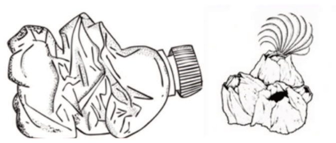


Figure S1. Additive content of virgin polyethylene pellets (A), virgin polyamide pellets (B) and beached pellets (C) (mean ± standard deviation; n = 3). For acronym interpretation, refer to Table 2. Note the two-orders of magnitude difference in additive concentrations observed between polyethylene pellets and both polyamide and beached pellets.

CHAPTER V



IMPACT OF BIO- AND CONVENTIONAL PLASTIC LEACHATES ON CIRRAL BEHAVIOR IN THE BARNACLE *AUSTROMINIUS MODESTUS*

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Abstract

Plastic, an essential but intrinsically harmful material in the modern world, poses a serious threat to marine organisms and ecosystem by leaching its additives and potential adsorbed chemicals. Despite the pivotal role of organismal behavior in connecting individuals to ecosystem functioning, the impact of plastic leachates remains barely explored. In this context, we conducted an assessment of the effects of polyethylene (PE), polyamide (PA), beached pellet (BP) and polylactic acid (PLA) leachates on the cirral behavior of the barnacle *Austrominius modestus*, examining the potential differences in both the mean and the variability of studied parameters. Given the potential influence of abiotic parameters such as temperature on leaching processes, we investigated the effect of the temperature on the plastic leachate toxicity by desorbing pellets at 10 or 20 °C. Our findings revealed that only PLA, a biobased and biodegradable plastic, significantly modified the mean of cirral behavior of *A. modestus*. Specifically, the cirral beat frequency (CBF) either increased or decreased when exposed to PLA leachates desorbed at 10 and 20 °C, respectively. In addition, the variability of the opercular opening time significantly increased under the influence of plastic leachates, irrespective of the polymer type. The toxicity of leachates exhibited variation based on polymer type, desorption temperature and observed traits. Surprisingly, PLA, despite its biobased and biodegradable nature, displayed unexpected toxicity, thereby challenging the commonly perceived environmental friendliness of bioplastics. While long been considered as noise, our results also underscore the necessity to include the variability of parameters in future studies.

Keywords: plastic leachates, bioplastics, interindividual variability, barnacle, cirral behavior

1. Introduction

Plastic is an essential material in our modern economy, optimally blending unparalleled functionality with low cost. Annually, over 390 million tons are produced (PlasticsEurope, 2022), and projections indicate a doubling of production within the next 20 years (MacArthur, 2017). However, this prolific use of plastic has led to a global challenge: plastic waste pollution (Geyer et al., 2017). Noticeably, intertidal environments, located in close proximity to urbanized areas, face a particular threat (Lebreton et al., 2019), especially given their crucial role in organisms' food reservoir and habitat (Sardá et al., 1998) and ecosystem functioning (Levin et al., 2001). Astonishingly, plastic constitutes up to 95 % of the coastal litter (Galgani et al., 2015), posing a significant risk to the delicate balance of these ecosystems.

Manufactured with a myriad of additives that confer distinct properties, plastics are inherently harmful (Lithner et al., 2011). For instance, plastic packaging items alone incorporate more than 4000 additives (Groh and Muncke, 2017), which are, in a vast majority, not chemically bound to the polymer matrix (Andrady, 2011; Koelmans et al., 2014). Consequently, these additives can easily leach into the environment causing primary leachates with documented various effects on organisms, such as reproduction and embryonic development impairments, behavioral alterations, cellular damages and mortality (see Delaeter et al. (2022) for a review). Plastic can also accumulate persistent organic pollutants (POPs) from the environment (Fries and Zarfl, 2012), subsequently released as secondary leachates (Delaeter et al., 2022). Numerous POPs have been identified in those leachates (e.g. PAH, PCB, PBDE, DDT, heavy metals; Kedzierski et al., 2018; Ogata et al., 2009) and concentrations can reach up to 6 orders of magnitude higher than in surroundings (Hermabessiere et al., 2017). Consequently, plastic can release a cocktail of hazardous additives and pollutants all over their lifetime, threatening intertidal organisms.

Behaviors stand as a critical lens through which we can assess the true impact of environmental stressors, such as plastic pollution on intertidal ecosystems. Unlike mortality, which is a more obvious (the most studied in plastic leachate literature; Delaeter et al., 2022) and less sensitive metric (Arnold et al., 2014; Little and Finger, 1990; Sih et al., 2010), behaviors are described as the frontline defense of organisms (Mench, 1998). Behavioral alterations can have far-reaching consequences, extending well beyond the individual fitness, shaping population and community dynamics (Brodin et al., 2014; Saaristo et al., 2018; White and Briffa, 2017; Zala and Penn, 2004). The responses of organisms to environmental challenges ultimately influence ecosystem functioning and evolutionary processes (Candolin and Rahman, 2023; Candolin and Wong, 2019; Duckworth, 2009; Saaristo et al., 2018; Wong and Candolin, 2015). Despite this pivotal role, the behavioral impacts of plastic pollution have been overshadowed in the literature. Only 4 studies have investigated the effects of plastic leachates on the behavior of marine invertebrates and highlighted modifications in the motility, the aggregation and the byssal thread production of mussels (Seuront et al., 2021), the vigilance and antipredator behavior of gastropods (Seuront, 2018), the copepods swimming behavior (Lehtiniemi et al., 2021) and the anxiety behaviors of crabs (see Chapter 3). Shading lights on the impacts of plastic leachates on the behaviors of a larger range of key invertebrates may provide a holistic understanding of the ecological repercussions of plastic pollution.

Inhabiting the highly exposed intertidal rocking habitats, barnacles play a pivotal role in the functioning of intertidal ecosystems. Their presence on rocky shore modifies environmental factors such as light, temperature, wave action, sedimentation and food availability (Barnes, 2000; Harley et al., 2006), thereby enhancing habitat complexity. Consequently, barnacles provide vital ecosystem services by offering a unique habitat for a diverse range of species that attach to their shells or inhabit the mud and debris accumulated between them (Stubbings, 1975). Even after death, the shells of barnacles continue to serve a crucial function, providing protection and refuge for various adult and juvenile invertebrates (Barnes, 1999). As true ecosystem engineers, barnacles influence community dynamics and ecosystem functioning. However, this ecological contribution may be under threat due to the accumulation of plastic debris in intertidal habitats. While an existing study have hinted at the potential of plastic leachates on barnacles, specifically on larval survival and reproductive success (Li et al., 2016), the behavioral effects of leachates have never been explored. Noticeably, barnacles are filter-feeders that depends on their cirri to feed (Anderson, 1994), breathe and reproduce (Crisp and Southward, 1961). Potential alteration of the cirral behavior may thus have broader consequences on individual and population survival, ultimately jeopardizing the functioning of barnacle-associated intertidal communities.

In this context, our study aims to fill the gap by investigating the impact of plastic leachates on the cirral behavior of the barnacle *Austrominius modestus*, the dominant species at our study site. We selected four distinct polymer types – polyethylene (PE), polyamide (PA), beached pellet (BP) and polylactic acid (PLA) – considering their prevalence in intertidal environments (PE and PA; Erni-Cassola et al., 2019; Pannetier et al., 2019), potential accumulation of pollutants (BP; Fries and Zarfl, 2012) and the emergence of biobased and biodegradable alternatives (PLA; Ali et al., 2023). Given that plastic leaching depends on natural processes such as UV irradiation, physical abrasion, oxidation and heat (Liu et al., 2020b), we hypothesized that leachate toxicity on barnacle cirral behavior will vary with the desorption temperature, and, accordingly, we investigated the toxicity of leachates from plastic pellets desorbed at two environmentally realistic temperatures (i.e. 10 and 20°C).

2. Materials and methods

2.1. Collection of barnacles

Barnacles *Austrominius modestus* were collected from Wimereux beach (France: 50°45'48''N, 1°36'10''E) in February 2023. Limpets covered of barnacles were sampled to avoid potential damages linked to the detachment of barnacles from their substrate, thereby avoiding the need for subsequent re-acclimatization to a new support. Once in the lab, limpets were conscientiously emptied and the shell were carefully rinsed with natural seawater. Limpets were then transferred in 500mL beaker and overnighted in running natural sea water in aquarium at field temperature (10°C). Two limpets were used to assess the behavior of 20 barnacles per treatments.

2.2. Leachate solutions preparation

Plastic leachate solutions were prepared at one concentration, that was calculated based on the exchange surface of the pellets to ensure comparability across treatments. The concentration was set at 50 g/L for PE pellets (4.0 x 2.0 mm, cylindrical shape). Subsequently, the concentrations for the other pellet types were determined based on the exchange surface of the pellets: 50 g/L of PE pellets corresponded to 53.3 g/L of PA (3.0 x 2.2 mm, ellipsoidal shape), 57.4 g/L of BP (3.5 x 3.1 mm, cylindrical shape) and 90.8 g/L of PLA (4.7 x 3.6 mm, ellipsoidal shape).

The potential impact of plastic leachates was investigated using 2 desorption temperatures. Consequently, five solutions (i.e. control, PE, PA, BP and PLA) were desorbed in incubator at 10 (i.e. field temperature) and at 20°C (i.e. environmentally realistic high temperature), resulting in a total of 10 solutions (with control solution named C_{10} and C_{20}, and leachate solutions named PE_{10}, PE_{20}, PA_{10}, PA_{20}, BP_{10}, BP_{20}, PLA_{10} and PLA_{20}; Fig. 1). After 24 h of desorption, pellets were sieved on a 2mm mesh and the 10 solutions were transferred to the controlled-temperature room set at 10°C for the exposure period (Fig. 1). To ensure that solutions reached the desire temperature for both desorption (10 or 20°C) or exposure period (10°C), the solutions were overnighted in the incubators (set at 10 or 20°C) before plastic pellet desorption and in the 10°C temperature-controlled room before exposure. Throughout these periods, solutions were consistently aerated using air pumps and control seawater solutions were prepared following the same procedure.

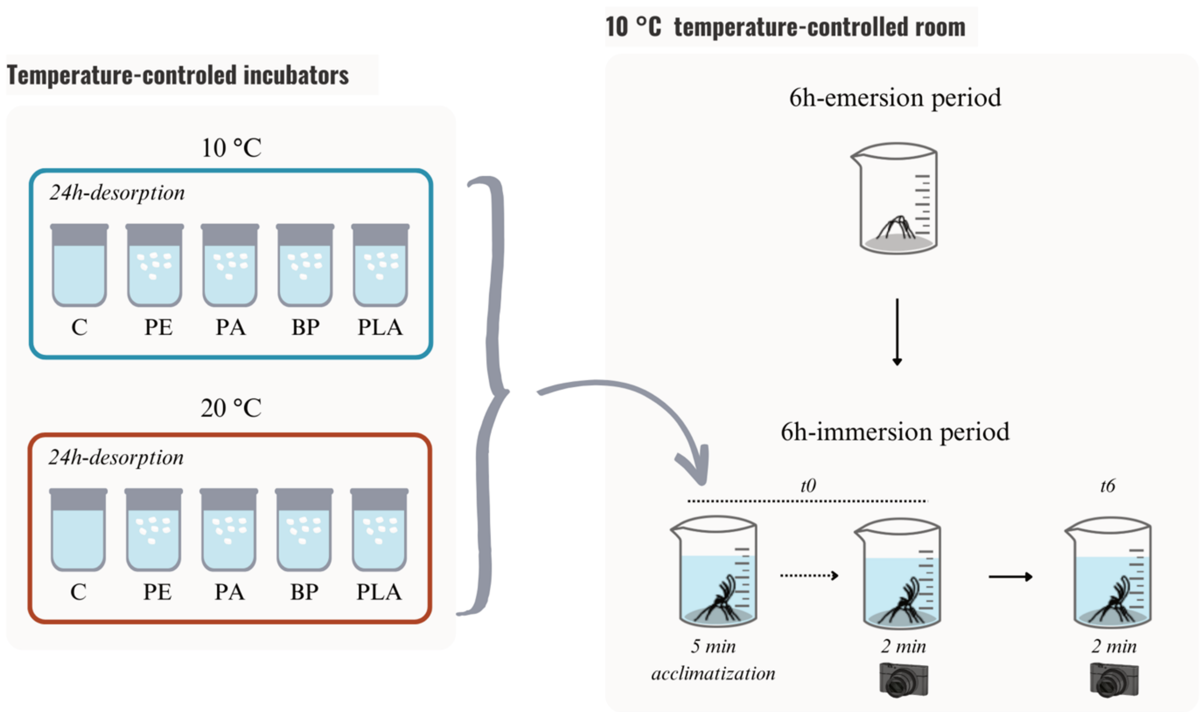

Figure 1. Representation of the experimental procedures.

2.3. Cirral behavioral assay and analyses

Barnacles were transferred (in their beaker) from the aquarium to a controlled-temperature room set at 10 °C for the exposure to leachates. To mimic environmental conditions, the beakers were emptied and barnacles underwent a 6h-emersion period. Following this emersion period, the beakers were filled with 400 mL of plastic leachate or seawater control solution to test the potential effect of plastic leachates on the barnacles' cirral behavior. Subsequently, a period of 5 minutes acclimatization was conducted, after which cirral behaviors were recorded for 2 minutes (period named t0) using a camera (Nikon Coolpix P7100). After 6h of immersion, cirral behaviors were again recorded for another 2 minutes (period named t6; Fig. 1). Throughout the 6h-immersion period, the beaker solutions were consistently aerated using air pumps, with meticulous attention paid to maintaining uniform aeration across all beakers.

The cirral behavior was assessed using two parameters: the cirral beat frequency (CBF) and the opercular opening. The CBF is defined as the time (in seconds) taken by barnacles to extend their cirri 5 times (Horn et al., 2021). Opercular opening was measured as the time they spent (in seconds) in an open state, engaging in either abbreviated or extended behaviors (see Box 1), during the 2-minutes video analyses.

Box 1. The different types of cirral behaviors
The cirral activity is categorized into 3 distinct types: (1) extended, (2) abbreviated and (3) closed behaviors (Nishizaki and Carrington, 2014). A comprehensive description of these cirral behaviors can be found in Crisp & Southward (1961) and are summarized here. Extended behaviors involve rhythmic beating of cirri implicating a movement of extension outside the opercular cavity followed by folding, either at normal or high speed (behaviors subsequently referred to as normal and fast beating, respectively). Abbreviated behaviors encompass testing and pumping activities. Pumping involves the opening and closure of opercular valves occur, accompanied by a slight lengthening of the cirri which however remain folded. Testing behavior is similar to pumping except that cirri stay inside the opercular valves. Closed behavior refers, in this work, to the isolation of opercular cavity from the external environment.
The cirral activity play a crucial role in the survival of barnacles, with extended behaviors contributing to feeding (although respiration can also occur), pumping being involved in respiration and microfeeding and testing being dedicated to the assessment of the condition and movement of water (Crisp and Southward, 1961).

2.4. Plastic pellet analyses

We assessed the behavior of barnacles exposed to leachates from four plastic pellet types: virgin polyethylene (PE, Materialix Ltd.), virgin polyamide (PA, Akulon F136-C1), beached pellets (BP, collected from Neufchâtel-Hardelot beach in France, 50°38'27.2"N, 1°34'37.4"E) and virgin polylactic acid (PLA, NatureWorks LLC, Ingeo^{TM} 4043D). The identification of the additives content of the plastic pellets was assessed using a CDS Pyroprobe 6150 pyrolyzer (CDS Analytical) in association with a GC-HRMS instrument (GC Trace 1310-MS Orbitrap Q Exactive, Thermo Fisher Scientific). Thermal desorption was performed (350 °C) to remove the potential additives from the samples. The samples were then separated using a Restek Rxi-5-MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with a cross-linked poly 5 % diphenyl-95 % dimethylsiloxane stationary phase (slip ratio: 1:5) and the acquisition was conducted on full-scan (FS) mode (m/z = 30.00000–600.00000). The resulting chromatograms were analyzed using Xcalibur and TraceFinder software for the identification of organic plastic additives among a selection of 57 additives (i.e. plasticizers, flame retardant, antioxidants and UVs stabilizers). The subsequent identification of the additives was based on their retention times, m/z values, and specific ions, which were compared with the chromatograms obtained from standard solutions of each additive.

2.5. Statistical analyses

As none of the behavioral parameters met the normality assumption, nonparametric statistics were employed consistently in this study. Kruskal-Wallis (KW hereafter) and Dunn tests were conducted to assess the potential impact of plastic leachates on barnacles' behavior. Potential differences in the variability were assessed using a Levene test. Statistical tests were conducted using the software RStudio (R 4.0.3).

3. Results

At t0, individuals displayed 3 types of behaviors, i.e. extended, abbreviated and closed behavior (see Box 1 for description), in all treatments. However, at t6, only abbreviated and closed behaviors have been observed. Although those behaviors are described as typical in aquarium captivity (Crisp & Southward, 1961), the beakers containing barnacles were not connected to running natural seawater during the 6h-immersion procedure and the concentration of food contained in the solutions must have decreased. Therefore, potential bias in cirral behavior linked to food concentration may have occurred at t6 and results have consequently not been analyzed.

Table 1. List of additives found in the pellets of different polymer depending on their function. Abbreviations means: tributyl Acetyl Citrate (ATBC), benzyl butyl phthalate (BBP), 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE153), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE154), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE183), butylated hydroxytoluene (BHT), bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), di-allyl phthalate (DAIP), phthalates dibutyl phthalate (DBP), bis-2-ethylhexyl adipate (DEHA), di(2-ethyhexyl)phthalate (DEHP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), diisodecyl phthalate (DIDP), diisoheptyl phthalate (DIHP), dimethyl phthalate (DMP), nonylphenol monoethoxylate (NP1EO), nonylphenol (NPs), tributyl phosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(2-chloroisopropyl)phosphate (TCPP), tris(1,3-dichloro-2-propyl)phosphate (TDCPP).

Polymer type	Additive function	Additives found in pellets
PE	Plasticizers	ATBC, BBP, DAIP, DBP, DEHA, DEHP, DEP, DIBP, DIDP, DIHP, DMP
	Antioxidants	BPA, BHT, BPF, BPS, NP1EO, NPs
	Flame retardants	BDE153, BDE154, BDE 183, TBP, TCEP, TCPP, TDCPP
PA	Plasticizers	DBP, DEP, DIBP, DMP
BP	Plasticizers	DEHA, DIBP
	Antioxidants	NPs
PLA	Plasticizers	DEP, DIBP, DMP

3.1. Chemical analyses of plastic pellets

The analysis of additives in PE pellets revealed the presence of various plasticizers, antioxidants and flame retardants. In contrast, PA and PLA pellets contained a smaller range of plasticizers and BP pellets were composed of two plasticizers along with one antioxidant (see results of additives analyzes in Table 1).

3.2. Impact of plastic leachates in the environmentally relevant context

In an environmentally relevant context at the moment of the experiment, i.e. both temperature of exposure and temperature of pellets desorption being 10°C, our findings revealed that plastic leachates had no significant impact on the opercular opening of barnacles but that the CBF was significantly modified (KW tests; Fig. 2a,b). When exposed to PLA leachates, *A. modestus* was extending more quickly its cirri compared to the control group and PE treatment (Dunn test; Fig. 2b). No significant differences have been observed between control group and PE, PA and BP treatments nor between PLA, PA and BP treatments (Dunn test; Fig. 2b).

No significant differences were observed in the variability of opercular opening and CBF between the control and the treatment groups (Levene test).

3.3. Impact of the pellets' desorption temperature

We investigated the potential impact of pellet incubation temperature on the toxicity of plastic leachates, by comparing the cirral behaviors of barnacles exposed to plastic leachates that were previously desorbed at 10 and 20°C. Higher desorption temperature had no effect on the opercular opening of barnacles as no significant differences were observed (KW test; Fig. 2c). CBF was still significantly impacted by PLA leachates. However, while CBF was significantly increased under PLA_{10}, it was significantly decreased under PLA_{20} (Dunn tests; Fig. 2d) and CBF was significantly different between PLA_{10} and PLA_{20} treatments (KW test). In addition, while no significant differences have been found in the variability of the CBF, the variability of opercular opening under was significantly increased under leachate exposure compared to the control group (Levene tests).

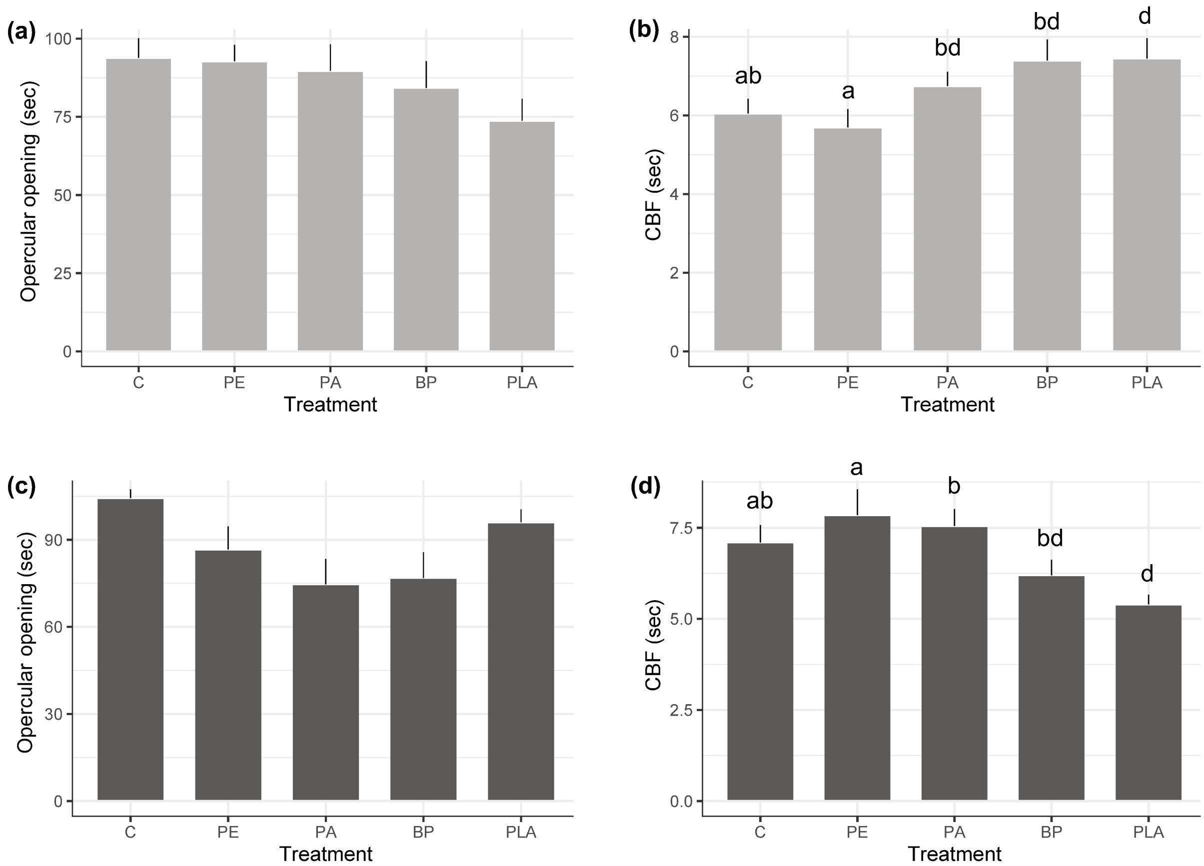

Figure 2. Impact of plastic leachates from polyethylene (PE), polyamide (PA), beached pellet (BP) and polylactic acid (PLA) on the cirral activity (a&c) and the cirral beat frequency (CBF; b&d) when the barnacles are exposed to plastic leachate solutions desorbed at 10 (a&b) and 20 °C (c&d). (C) is the control group and the letters illustrate significant differences (Dunn tests).

4. Discussion

Previous studies on the impact of plastic particles on barnacles revealed that plastic leachates significantly decreased larval survival and reproductive success (Li et al., 2016). However, another study concluded to a non-toxicity of virgin PS particles on the cirral beat frequency (CBF; Yu et al., 2020). Given the polymer-dependency of plastic toxicity, we investigated the impact of plastic leachates from various polymer type and revealed significant impact on the cirral activity of *Austrominius modestus*.

4.1. Impact of plastic leachates in environmental condition

For experiments conducted in environmentally relevant conditions (i.e. desorption of plastic pellets and exposure at 10°C), our results demonstrated a significant increase in the CBF under exposure to PLA leachates only. Similar behavioral changes have been previously observed in the literature when barnacles were exposed to diesel oil (López and López, 2005). Authors have suggested that an increase in activity is correlated with an elevation in metabolism. Indeed, when facing contamination, organisms often experience enhanced metabolic demand to activate detoxification pathways (White & Briffa, 2017). Notably in barnacles, there is evidence linking cirral activity to oxygen consumption (Anderson and Southward, 1987; Newell and Northcroft, 1965). Such shifts in metabolic rates may reduce the available energy for other physiological processes, such as growth (Toro et al., 2003). In this case, an increase in the barnacle opercular opening (as observed in López and López, 2005) may compensate for the increase in energy costs; however, our findings did not reveal such modifications in opercular activity. Consequently, plastic leachates contamination could lead to a rapid decline in barnacle condition, seriously compromising their survival.

4.2. Impact of the desorption temperature on the plastic leachates toxicity

As plastic leaching is influenced by processes such as UV irradiation, physical abrasion, oxidation and heat (Liu et al., 2020b), we examined the toxicity of leachates after desorption at 20°C, noting significant differences in their impacts. Indeed, while desorption at 10°C of PLA leachates led to an increase in CBF, the opposite effect was observed when desorption occurred at 20°C, resulting in a significant decrease in the CBF of *A. modestus* when exposed to PLA_{20} compared to the control. Supposing that higher leachate concentration, hence potential toxicity, explanation for such opposite effects may lie in the threshold tolerance of contaminants. Low concentrations may induce a stress response related to an increase in metabolic demand, while higher concentrations could exceed the tolerance threshold and impair organisms' performances. Furthermore, the higher incubation temperature might have led to the release of additional additives absent in the PLA_{10} solution, creating a cocktail of chemicals inducing opposite effect on the barnacle's CBF.

Notably under leachates desorbed at 20°C, although there was no impact on the opercular opening mean, a significant increase in the inter-individual variability was observed. Inter-individual variability, although long been considered as noise, is crucial for organisms. Related to behavioral plasticity, variability enables organisms to adapt to the intrinsically variable environment, such as intertidal habitats, acting as a buffering effect at population and ecosystem levels (Wolf and Weissing, 2012). In the present work, the response of *A. modestus* to plastic leachate pollution varied from one individual to another, with some increasing the time spent with the opercular open while others spent more time closed. Changes in variability have previously been associated to decrease in metabolic rate (i.e. oxygen consumption) in *Daphnia magma* (Nikinmaa et al., 2019) and might consequently been linked to stress level in the organisms. However, evidence of such relationship requires further investigation and should be considered in future ecotoxicologic studies (Saaristo et al., 2018).

4.3. Polymer-dependency and bioplastic toxicity

Our findings confirm the polymer-dependency of the impact of plastic leachates, as previously observed by Li et al. (2016), and the difference in polymer composition were corroborated by the pellets analyses. Surprisingly, PLA leachate was more toxic than PE, BP and PA despite PLA pellets not containing the highest number of additives. However, it is important to note that the presence of additives on pellets does not necessary lead to desorption and presence in the leachates. In addition, our chemical analyses encompassed the search for 57 additives, and the observed effects are likely induced by untargeted molecules.

PLA is a bioplastic, both biobased and biodegradable, use as substitute for PE and PET (Ali et al., 2023). Bioplastics have been increasingly developed in recent year to address environmental plastic pollution, aiming to reduces both the use of fossil fuel materials and waste accumulation. Recent studies have however highlighted their toxicity on marine organisms, revealing a similar or even greater toxicity then conventional plastic (Balestri et al., 2019, 2017; Green, 2016; Green et al., 2016; Magara et al., 2019; Menicagli et al., 2019; Zimmermann et al., 2020). For example, PLA had a stronger adverse effect on oyster respiration rates compared to PE exposure (Green, 2016) and induced maladaptive behaviors and increased activity level to disturbance in crabs whereas PA did not (see Chapter 3). Even more concerning is the evidence of higher toxicity of bioplastics compared to conventional ones. For instance, PLA induced detrimental impacts on phytoplankton assemblages, whereas PS exposure showed no such effect (Yokota and Mehlrose, 2020). Furthermore, we specifically chose these PLA pellets for their ‘safety’ characteristics, described as safe for food packaging and compostable (i.e. environmentally friendly), yet our findings do not provide reassurance regarding the potential increase in the use of bioplastics as a solution to the worldwide plastic pollution issue.

5. Conclusion

Our study revealed significant adverse effects of plastic leachates on the cirral activity of the barnacle *Austrominius modestus*. The toxicity of leachates varied with polymer type, desorption temperature and observed traits. Notably, PLA, despite being biobased and biodegradable, exhibited unexpected toxicity, thereby challenging the perceived environmental friendliness of bioplastics. This underscores the necessity for further research into the ecological implications of alternative materials, as they may pose similar or greater risks to marine organisms, thus ultimately threatening the ecosystemic balance.

In addition, the consideration of variability in our assessment revealed further insights into the toxicity of plastic leachates not apparent in the parameters means. Acknowledging the importance of variability in organisms’ survival in highly variable environments, future investigations on plastic pollution impacts should incorporate this aspect for a more comprehensive understanding.

Acknowledgment

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GENERAL DISCUSSION & CONCLUSION

1. Literature review: gaps identification and experimental choices

1.1. Gaps identification

Plastic is a ubiquitous component of the modern world generating millions of tons of waste (Geyer et al., 2017) that accumulate in marine environments and increasingly threaten the delicate ecosystem balance (Jambeck et al., 2015; Lebreton et al., 2019). Since the onset of its mass production in the 1950s, the literature on plastic pollution has grown exponentially, exceeding 4,000 papers published annually since 2019. However, our literature survey revealed only 26 papers addressing the impact of plastic leachates on invertebrates (Delaeter et al., 2022). These studies highlighted effects on various individual processes, including survival (Bejgarn et al., 2015; Capolupo et al., 2020; Gardon et al., 2020; Gewert et al., 2021; Ke et al., 2019; Li et al., 2016; Trestrail et al., 2020), reproduction (Capolupo et al., 2020; Koski et al., 2021; Li et al., 2016), embryonic development (Cormier et al., 2021; Martínez-Gómez et al., 2017; Nobre et al., 2015; Oliviero et al., 2019; Piccardo et al., 2020; Rendell-Bhatti et al., 2021), oxidative stress (Capolupo et al., 2021), behavior and cognition (Seuront, 2018; Seuront et al., 2021). Nevertheless, several gaps have been identified in the leachate literature, highlighting both disparities and lacks in the organisms considered, and discrepancies between the polymers studied and their abundance in the marine environment, especially in intertidal habitats. Furthermore, a crucial lack of methodological consistency throughout the literature was stressed. Taken together, these findings suggest that the current literature is not sufficient for the understanding of the environmentally relevant impact of plastic leachates, limiting our ability to efficiently address the plastic pollution plague. In light of these conclusions, we subsequently made experimental choices to investigate the impact of plastic leachate on the behavior of benthic intertidal organisms.

1.2. Experimental choices

1.2.1. Choice of polymer types and leachates concentration

Plastics, inherently harmful due to the cocktail of additives leaching from their surface, release what we referred to as 'primary leachates' (Delaeter et al., 2022). Additionally, plastic items accumulate anthropogenic pollutants, such as heavy metals, PCB, PAH, DDT, PBDE (Kedzierski et al., 2018; Ogata et al., 2009), potentially released as 'secondary leachates' (Delaeter et al., 2022). Given the staggering 5×10^9 tons of plastic already accumulating in the environment (Geyer et al., 2017), the 4 to 12×10^6 tons entering oceans every year (Jambeck et al., 2015), and both the ever growing accumulation of plastic debris in intertidal ecosystems from both marine and terrestrial origins (see e.g. Browne et al. (2011) and Galgani et al. (2015)) and the widely acknowledged contamination of coastal waters by a range of chemical compounds such as heavy metals, POPs and pesticides, intertidal organisms are fundamentally exposed to both primary and secondary leachates.

To include both types of leachates in our experiments, we studied the impact of **beached pellet** alongside with 3 specifically chosen virgin polymer types:

- **Virgin PE pellets**: though PE is the most widely represented in the literature (ca. 16 % of the research effort; Delaeter et al., 2022), it is nonetheless the most widely produced (PlasticsEurope, 2022) and found in the environment (Erni-Cassola et al., 2019). Notably, only one study has investigated the toxicity of PE (from virgin LDPE granules and recycled LDPE vegetables packaging) on behavior (copepod swimming behavior; Lehtiniemi et al., 2021), showing no leachate toxicity.
- **Virgin PA pellets**: previously identified as a greatly under-represented polymer in the plastic leachate literature compared to its environment occurrence (Delaeter et al.,

2022), PA virgin polymer was included in our experiments. It was specifically chosen due to its widespread use (PlasticsEurope, 2021), e.g. clothing, fishing nets.

- **Virgin PLA pellets**: current efforts to address plastic pollution focus on finding solutions to minimize environmental impact, with bioplastics taking center stage. As biobased and/or biodegradable alternatives, they are considered a solution to both resource management and environmental pollution. However, the literature noticeably contains examples of bioplastics being as toxic as their fossil fuel counterparts (Green, 2016; Green et al., 2016; Yokota and Mehlrose, 2020). PLA, a substitute for PE and PET (Ali et al., 2023), was chosen for its biosourced and biodegradable nature, widespread use in food packaging and being among the most produced and studied biopolymer.

To address methodological inconsistencies and facilitate comparisons between studies, we made decisive choice regarding the unit of measurement applied for the experiments. Unlike most studies somehow indistinctively dealing with number, weight or volume of plastic beads, we chose to proceed by exchange surface. This unit allows us to compare the toxicity of plastic leachates, irrespective of bead size and weight. PE was chosen as the reference polymer, being the most represented in the literature (Delaeter et al., 2022). We calculated the surface area of our beads based on their shape (cylindrical or oblong) and size, determined the number of beads needed to achieve the desired weight and converted this result into total exchange surface area per gram of beads for each bead type. Finally, we calculated the amounts of beads required for PA, BP and PLA for 10 and 50 g L^{-1} PE, based on equivalent exchange surface.

1.2.2. Choice of experimental candidates

Our choice of experimental species stemmed from the identification of a substantial research gap within the studied organisms in the plastic leachate literature, focusing specifically on intertidal environments due to the considerable accumulation of plastic in these areas (Galgani et al., 2015). We strategically pinpointed key species to ecosystem functioning (from both intertidal rocky shores and mudflats), which had either been minimally studied or entirely overlooked in the current plastic leachates literature. Despite crustaceans representing 61 % of the research effort, no investigations were conducted on crabs and only one study focused on barnacles. These organisms play, however, pivotal roles in intertidal ecosystems, contributing to trophic food web (Boudreau and Worm, 2012; Little and Finger, 1990; Raffaelli and Hawkins, 1996), engaging interactions with other species (Richards and Cobb, 1986; Rossong et al., 2006), and/or providing juvenile refuge and habitat (Stubbings, 1975). They consequently allow shaping the structure and function of benthic intertidal communities (Boudreau and Worm, 2012) and allow the presence of a great variety of animals on rocky shores (Barnes, 2000; Harley, 2006; Stubbings, 1975). Similarly, foraminifera despite their critical role in both bioturbation and bio-irrigation of sediment (Cedhagen et al., 2021; Deldicq et al., 2023; Orvain et al., 2004), their central trophic position in food web (Chronopoulou et al., 2019; Gooday et al., 1992; Nomaki et al., 2008; Wukovits et al., 2018) and their role as bio-indicators of various pollutants (Armynot du Châtelet et al., 2004; Burone et al., 2007; Ernst et al., 2006; Pawlowski et al., 2014; Vidović et al., 2014) were studied in only one study (Langlet et al., 2020). Consequently, the Asian shore crab *Hemigrapsus sanguineus*, the barnacle *Austrominius modestus* and the foraminifera *Haynesina germanica* were selected as candidate for assessing the effect of plastic leachates on the behavior of benthic intertidal organisms.

2. Behavioral impact of plastic leachates and underlying physiological and morphological alterations

Behaviors are of utmost importance for organisms' survival, being engaged in foraging for finding food or a mate, escaping predators or uncomfortable places, competing for territory, seeking refuge or even migrating. Behaviors not only influence individual outcomes but also shape population and community dynamics, and ultimately impact ecosystem functioning and evolution (Candolin and Rahman, 2023; Candolin and Wong, 2019; Duckworth, 2009; Saaristo et al., 2018; Wong and Candolin, 2015). However, literature on plastic leachates impacts on individual behavior is very limited (Lehtiniemi et al., 2021; Seuront, 2018; Seuront et al., 2021), though behavior is much more sensitive to assess stress than more traditional parameters such as mortality (Arnold et al., 2014; Little and Finger, 1990; Sih et al., 2010). In this context, this thesis aims to contribute to this gap by exploring the effects of plastic leachates on the behavior of benthic intertidal organisms, specially focusing on the crab *H. sanguineus*, the foraminifera *H. germanica* and the barnacle *A. modestus*.

The findings revealed significant modifications in anxiety-related, motion and cirral behaviors of these organisms in response to plastic leachates through adoption of maladaptive behaviors and both increase or decrease in activity. First, enhanced activity was consistently observed in crabs (i.e. increase activity in black area; Chapter 3), foraminifera (i.e. increased motion behavior and appearance of erratic behavior; Chapter 4) and barnacles (i.e. increased cirral activity; Chapter 5) when these organisms were exposed to plastic leachates. Those behaviors have been linked to potential increase in stress level underlying a modification in metabolic processes. Indeed, increased activity have been identified as a response to organisms to cope with higher metabolic demand needed for the activation of detoxification pathways (White and Briffa, 2017). Noticeably, increase in metabolic rate, is inducing a decrease in the energy available for other processes, such as growth (Toro et al., 2003) or risk avoidance. The subsequent requirement of energy can be compensated, for instance by an increase in food intake (López and López, 2005), but if not, such as what have been observed in barnacles (i.e. the increase of cirral beat frequency (CBF) was not associated by an increase in opercula opening; Chapter 5), then the survival of the organism is jeopardize as its condition may degrade rapidly.

Maladaptive behaviors have also been induced by exposure to plastic leachates. Significant increase in the number of scototactic positive crabs (i.e. preferring dark environments that are associated to safe places) entering a white area (instead of a black one) while disturbed by the removal of the acclimatization cage have been demonstrated (with no associated effect on the scototacic behavior; Chapter 3). Moreover, the tortuosity of foraminifera trajectories has been significantly modified under leachates exposure that may potentially resulted in a non-adapted foraging strategy (Chapter 4). Alterations of such organisms' behavior are typically observed in anxiety-related studies (Maximino et al., 2010a), and are associated to modification in stress, impairments in metabolism or 'info-disrupting' in the gathering and processing of perceived information (Lürling and Scheffer, 2007). Such maladaptive behaviors are impairing the individual fitness by rendering the organism more vulnerable. For example, the maladaptive behavior of *H. sanguineus* is potentially increasing its predation risk and the modification in the trajectory of *H. germanica* is likely to decrease its efficiency in searching for food.

Exposure to plastic polymers also induce a decrease in the activity index (i.e. the time allocated to movement) of foraminifera (Chapter 4) and in CBF of barnacles (when exposed to leachates desorbed at 20°C; Chapter 5). Two explanations may arise to explain these a priori contradictory effects. First, morphological damages such as impairment in foraminiferal pseudopods may have occurred (note that in this case it can be related to cytoplasmic alterations (Nigam et al., 2009; Saraswat et al., 2004) or modifications in metabolic rates (Cedhagen and Frimanson, 2002)). A decrease in organisms' activity may also be related to damages in physiological processes at one or more levels in metabolism, hormonal functions, sensory system and neurological processes (Scott and Sloman, 2004; Sih et al., 2010).

Although further investigations are needed to deepen the reasons underlying behavioral alterations, this work have straightened the fact that the impact of plastic leachates are trait and species dependent, affecting organisms playing pivotal roles in the ecosystem functioning (even resistant species such the invasive *H. sanguineus*) and threatening its delicate balance. While only the response to disturbance and activity in black, and the cirral beat frequency should be targeted for future investigations on *H. sanguineus* and *A. modestus* respectively, every variable considered in the description of the motion behavior of *H. germanica* has been sensitive to leachate exposure. Therefore, future investigations should prioritize the study of foraminifera as an indicator of plastic leachate pollution.

3. Inter-individual variability: a not so noisy parameter

Typically considered as noise, variability is still represented as 'error bar', 'standard error' or 'confidence intervals'. However, its significance extends far beyond, being an underappreciated supplementary source of information of the impact of environmental changes on organisms. Variability influences interactions between species, distribution within habitats, species dispersion and invasion, transmission dynamics, ecosystem processes and ultimately allow stability, resilience and persistence of populations (Wolf and Weissing, 2012). Recognizing the insights that behavioral variability under contamination can offer is crucial for understanding the impact at both population and ecosystem levels. Inter-individual variability provides individuals from the same population to adopt different intensities of a behavioral trait. This intrinsic variability is closely related to the behavioral plasticity, allowing for the adoption of a certain intensity of a behavior belonging to a continuum between a maximum and a minimum (Biro et al., 2013; Réale et al., 2007, Fig. 1). At the individual scale, phenotypic variability is at the base of individual personality, characterizing the behavioral trait of an individual that is maintained through time and across different context (Wolf and Wessing, 2012; Fig. 1). At the population scale, the range of the behavioral continuum depends on the differences between individual personalities, and behavioral plasticity acts as a buffering effect at population and ecosystem level (Wolf and Weissing, 2012).

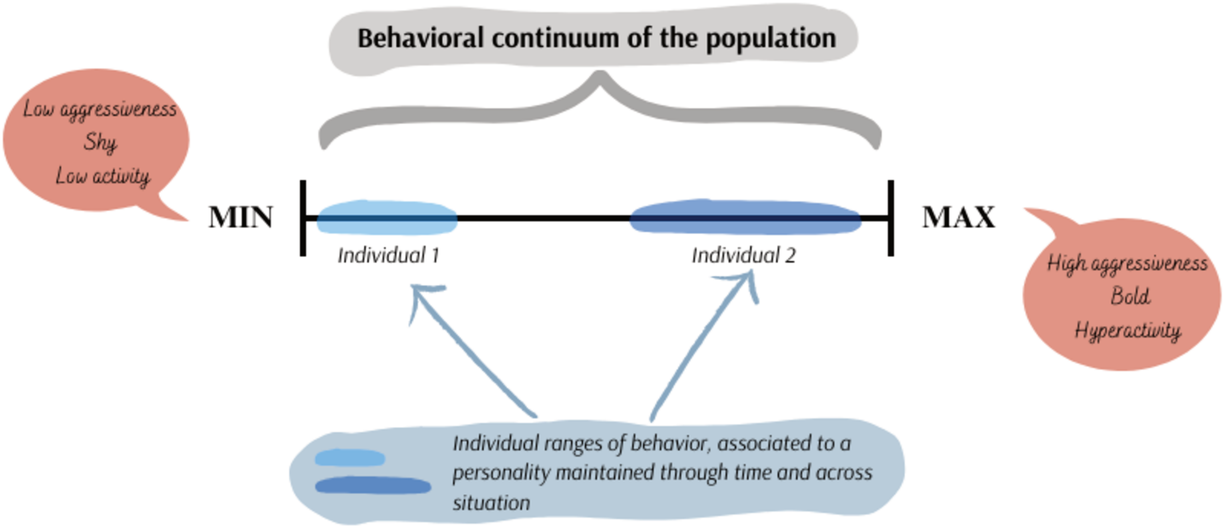

Figure 1. Graphical representation of the behavioral plasticity and individual personality.

The behavioral plasticity of a population is intrinsically related to environmental stability (Klopfer and MacArthur, 1960): the more variable the environment, the wider the continuum of behavioral traits. Studying benthic intertidal organisms, from highly variable habitats, unsurprisingly revealed high variability throughout all experiments (coefficient of variation reaching up to 123 % in control group; Chapter 3). To assess the potential impacts of plastic leachates on organisms' ability to cope with their variable environment, I conducted additional Levene tests for each parameter (results are presented in Box 2). These tests revealed a noteworthy impact of plastic leachates on interindividual variability. Interestingly, certain behavioral parameters, despite not exhibiting a significant change in their mean values due to plastic exposure, demonstrated significant differences in their variability. These parameters include crab startle time, scototaxis and thigmotaxis after exposure to some leachates from plastic pellets (Annexe 2 Fig. 1; Box 2). Similarly, Nakinmaa et al. (2019) observed a significant decrease in the variability in oxygen consumption of *Daphnia magna* exposed to crude oil, whereas no difference was observed in the mean oxygen consumption. A decrease in variability can jeopardize the survival of intertidal organisms, hindering their ability to cope with environmental changes and ultimately threatening population survival.

Box 2. Tests of behavioral variability

Levene tests have been conducted on each behavioral parameters assessed from crabs, foraminifera and barnacles' experiments to compare the variability in control compared to treatment groups. Results are represented in Annexe 2.

While no significant differences have been observed in the experiments on the impact of leachates from surgical masks, startle time, scototaxis and thigmotaxis variability was significantly impacted in the experiment on the impact of leachates from plastic pellets depending on polymer type. The variability of thigmotactic and scototactic behaviors were significantly decreased when crabs were exposed to PLA and PE leachates, respectively, compared to the control groups. Under PE and PA leachates, the variability of the startle time significantly decreased compared to the control. On the contrary, an increase in startle time variability was observed when crabs were exposed to PLA leachates. While the standard deviation of startle time under BP leachates was ca. 91, surprisingly no significant difference was found in the variability. This must be explained by the behavior of one individual, displaying a very high startle time, that is likely to drag the deviation from average hence matching the values' repartition of the control group.

The variability of the activity index significantly decreases and increases when foraminifera were exposed to BP and PE leachates, respectively.

Finally, the variability of the opercular opening duration was significantly increased when barnacles were exposed to leachates from pellets desorbed at 20°C, no matter the polymer type.

Notably, the variability observed in thigmotaxis is significantly reduced when *H. sanguineus* is exposed to PLA leachates. In this experiment, we observed more individuals spending 100

% of their time in contact with the walls of the arena (i.e. more than 80 % compared to 58 % in the control group). Increased thigmotactic behavior has been previously associated with increasing level of anxiety (Maximino et al., 2010a), suggesting that variability may also be a proxy of stress. This assumption is further corroborated be the increase in risk-taking behavior of crabs exposed to PLA leachates (i.e. the increased number of individuals entering a white instead of a black area). Increase or decrease in variability might thus be the reflect of decrease or increase in stress level, respectively. Therefore, variability, likely more sensitive than the mean, may be considered as an early indicator of behavioral alterations and should be considered in future investigations.

4. Polymer-dependency, cocktail and dose-effect

A noticeable characteristic of the plastic leachates contamination observed in every experiment, that noticeably corroborate the findings of the literature review (Delaeter et al., 2022; Chapter 1), is the polymer-dependency of the behavioral impacts. Such dependency is explained by the composition of plastic pellets that vary with polymer type, the intended final product and the manufacturer ‘recipe’. The observed toxicity of plastic leachates was not necessarily in accordance with the results of chemical analysis of plastic pellets composition. Indeed, while PE contained far more additives than PA, BP and PLA pellets, PLA leachates were toxic to barnacles CBF whereas other leachates were not (Chapter 5), and BP affected more importantly foraminiferal behavior than PE and PA (Chapter 4). Similarly, leachates from surgical masks did not induce any impact on means or variability of crab behaviors whereas PE, BP and PLA did (Chapters 2 and 3). Such discrepancies may, however, be explained by the fact that pellet composition is not necessarily identical to leachate one, as leaching capacities may vary between additives. Furthermore, we targeted 57 additives in our chemical analysis of pellets and untargeted chemicals are likely to be responsible for toxicity. Specifically, BP can have accumulated persistent organic pollutants (POPs) during its stay in environment, subsequently releasing as secondary leachates. POPs include chemicals such as heavy metals, PAH, PCB, PBDE (Kedzierski et al., 2018; Ogata et al., 2009), that are known highly toxic (Weis, 2019).

Our findings further demonstrated that the toxicity of plastic leachates is not as trivial as 1 + 1 = 2. While higher damages were logically observed under high concentration (higher reduction of the initial speed have been observed when exposed to higher concentration of PA; Chapter 4), opposite responses between low and high doses have also been observed in foraminifera and barnacles. Under PE and BP exposure, low concentrations led to a decrease in the initial speed whereas high concentrations increase the initial speed (Chapter 4). Similarly, while PLA leachates desorbed at 10°C induced an increase in barnacles CBF, PLA leachates desorbed at 20°C (and supposed more toxic) induced a decrease in barnacles CBF (Chapter 5). Although surprising, such effects can be related to what is called 'hormesis', i.e. an adaptative (even positive) response of cells and organisms to a moderate dose of contaminant inducing favorable response at such dose (Mattson, 2008). For example, moderate dose of cannabis led to a decrease in pain whereas high dose induced an increase in the felt pain in human (Wallace et al., 2007). Similarly, diazinon had contrary effects on the motor activity of the rat ileum, increasing contraction at low dose and decreasing it at high concentration (Trailović et al., 2017). Linked to endocrine disruption, unexpected harmful effect at low dose can arise because of contaminant's chemical similarity with hormones. Indeed, the authors hypothesized that at high dose, diazonin had a blocking effect on the receptors they are binding at low concentrations by binding additional accessory site.

In addition, the composition and resulting toxicity of leachates also depend on abiotic parameters, e.g. biodegradation, heat, UV irradiations, physical abrasion or oxidation (Liu et al., 2020b). Indeed, when incubated under different temperature (i.e. 10 and 20°C) the plastic leachates induced different, even opposite, impact of the cirral beat frequency of the barnacles (Chapter 5), suggesting different composition of the 20°C-leachate compared to the 10°C. Higher temperature being suggested to increase leaching processes (Liu et al., 2020b), the opposite response observed under 20°C-leachate exposure may be due to higher concentration of desorbed additives alongside with leaching of additional additives that are likely to need higher temperature to desorb from plastic pellets.

Taken together, our findings suggests that organisms are likely to face a cocktail of chemicals that have their own effect when alone but very unpredictable ones when added together rendering the relevance of the conclusion of the effect of plastic leachates very challenging if not impossible.

5. Alternatives to conventional plastics: is it the realistic solution?

Bioplastics have been increasingly developed in recent year to address the environmental plastic pollution issue. Today there is an alternative for almost every conventional plastic material and corresponding application (European Bioplastics, 2023). Bioplastics have been developed in order to respond to several objectives: bio-based plastics have the objective to use renewable carbon sources instead of fossil sources and biodegradable plastics have the objective to degrade when inadvertently emitted to the environment, thus reducing the plastic wastes (Lambert and Wagner, 2017). However, biopolymers present limitations concerning aging (e.g. water and oil resistance, durability) and as such their composition are chemically modified to improve their stability by the adding of chemicals (Sagnelli et al., 2017), which fundamentally poses toxicological issues. Noticeably, impacts on oxidative stress response (Magara et al., 2019), composition of communities (Green et al., 2016), algal growth (Balestri et al., 2017), plant germination (Balestri et al., 2019) and coastal dune vegetations have been identified.

PLA is a biobased and biodegradable bioplastic, use as substitute for PE and PET (Ali et al., 2023). Noticeably, our findings demonstrated the toxicity of PLA on *H. sanguineus*, inducing similar maladaptive crab behavior than conventional plastic (Chapter 3). Similar results have been observed in the literature: PLA having a stronger adverse effect on oyster respiration rates compared to PE exposure (Green, 2016). Even more concerning is the evidence of higher toxicity of PLA compared to conventional ones. Indeed, the only polymer having a detrimental effect on the CBF of barnacles was PLA (Chapter 5) in agreement with previous findings (Yokota and Mehlrose, 2020). For instance, PLA induced detrimental impacts on phytoplankton assemblages, whereas PS exposure showed no such effect (Yokota and Mehlrose, 2020). Although we specifically chose these PLA pellets for their ‘safety’ characteristics, described as safe for food packaging and compostable (i.e. environmentally friendly), yet our findings do not provide reassurance regarding the potential increase in the use of bioplastics as a solution to the worldwide plastic pollution issue.

As toxic as conventional polymers, it seems that they are not more interesting in the environmental accumulation point of view. Indeed, mainly produced for their degradability capacities, the reality of biodegradable polymers is far from the expected properties. Many of the bioplastics labelled biodegradable actually do not degrade under natural environmental conditions (Napper and Thompson, 2019). The degradation process depends not only on polymer properties but also on environmental conditions such as light, temperature, humidity, pH, microorganisms, enzyme and concentrations (Manfra et al., 2021), conditions that environment not meet. Furthermore, PLA generates a number of microparticles almost 3 times greater to what is generated by fossil fuel plastics (Yang et al., 2022). Bioplastics can also adsorb higher contaminant levels than non-degradable plastics (Gong et al., 2019; Shi et al., 2023; Torres et al., 2021) and readily leach their additives (Quade et al., 2022), rendering them equally harmful to aquatic organisms as fossil fuel plastics. Altogether those findings further stress that, despite their marketed eco-friendliness, bioplastics may not be the solution to the plastic pollution issue, emphasizing the importance of including bioplastics in studies that investigate the impacts of plastics and their leachates on marine organisms.

6. General conclusion

Based on identified gaps in the plastic leachates literature, the thesis focused on the behavioral responses of the crab *Hemigrapsus sanguineus*, the foraminifera *Haynesina germanica* and the barnacle *Austrominius modestus* to exposure of leachates from various polymer types. Significant modifications in the anxiety-related, the motion and the cirral behaviors have been identified, emphasizing the trait, polymer and dose-dependency of plastic leachate effects. Notably, PLA, a biobased and biodegradable polymer, leachates induced as many or even more behavioral modifications than leachates from conventional polymers, seriously questioning their use as 'eco-friendly' alternatives to traditional plastics.

Behavioral changes, likely to be induced by alterations of physiological and morphological processes, may have detrimental effects at the individual level, potentially leading to cascading impacts on population and community dynamics, ecosystem functioning and evolutionary processes. However, it is important to note that organisms in the marine environment face a complex mixture of contaminants, challenging the formulation of general conclusions.

PERSPECTIVES

Impact of the quality of the organism's habitat on its sensitivity to contamination

The behavior of individuals is dependent on their surrounding environment and driven by physiological processes. Noticeably, a range of studies have demonstrated that individuals of the same species from different populations exhibit different metabolic traits depending on the level of contamination of the habitat. For example, *Hemigrapsus edwardsi* from polluted sites had a lower heart rate and the population showed higher interindividual variability than their counterparts from less polluted sites (Depledge and Lundebye, 1996). Hence, it is warranted to inquire into how individuals, inhabiting environments with varying degrees of pollution, react to identical contamination.

Notably, Langlet et al. (2020) did not observed any impact of PP leachates on the behavior of the benthic foraminifera *Haynesina germanica*, collected from the Boulogne-sur-Mer harbour (France). However, we observed significant effects depending on the trait, polymer and dose tested when *H. germanica* was exposed to PE, PA and BP leachates (Chapter 4). Our foraminifera were collected in the Bay of Authie, and despite the need for further analyses to define the pollution levels of these two environments, it is likely that the Bay of Authie is much less contaminated. In this context, two hypotheses can be formulated: either PP leachate is not toxic compared to those from PE, PA and BP, or foraminifera from the Boulogne-sur-Mer harbour exhibit less sensitivity. Indeed, inhabiting a particularly polluted area, this population of *H. germanica* may have developed a resistance to pollution and be less sensitive than the population in the Bay of Authie.

The same PP beads (from the same batch) were also used in Seuront (2018) and led to an impairment of vigilance and antipredation behaviors in the gastropod *Littorina littorea*. Therefore, it seems that the two populations of *H. germanica* exhibit different sensitivities to exposure to plastic leachates and investigated the impact of plastic leachates on the population coming from both sites may confirm this hypothesis.

What future for ecosystems under the threat of astonishing plastic pollution?

Despite the current prominence of plastic pollution in policy discussions and the implementation of various solutions, our study reveals that bioplastics may not offer a sustainable solution for the future. Beyond their lack of biodegradability, these alternatives prove to be as (if not more) toxic than conventional polymers (Balestri et al., 2019, 2017; Green, 2016; Green et al., 2016; Magara et al., 2019; Menicagli et al., 2019; Zimmermann et al., 2020; see also Chapters 3 and 5). Moreover, recent investigations suggest that some proposed alternatives to plastic may not effectively address the challenges of environmental contamination and harm organisms. For instance, alternatives derived from secondary fibers often contain residuals inks and other chemicals due to incomplete deinking during the pulping process (Liu et al., 2020a), raising significant concerns about their environmental safety.

In addition to concerns about the toxicity of the end product, there are notable issues associated with their manufacturing. For example, the production of PLA, composed of starch, not only comes at a higher cost than conventional plastics but also conflicts with societal demands for feedstock (Koh et al., 2018). Similarly, while glass resolves the single-use plastic issue by not releasing any toxic components, its manufacturing has more substantial environmental impact compared to plastic (Humbert et al., 2009). Indeed, a study indicate that plastic production is associated with a 14 to 27 % reduction in primary energy, 28 to 31 % in global warming, 31 to 34 % in respiratory inorganics and 28 to 31 % in terrestrial acidification/nutrification compared to glass production (Humbert et al., 2009).

The question then arises: is there a genuinely convincing alternative to plastic? The answer is not straightforward, as some seemingly ‘eco-friendly’ solutions may not be as environmentally friendly as they appear, considering both direct impact of the end product and the broader consequences of its production and cultivation. Emerging alternatives based on plant-based raw materials, such as sugar cane, bamboo fiber (Liu et al., 2020a) or coconut husk (Leow et al., 2022), could potentially offer a starting point for addressing the challenge of finding a sustainable alternative to plastic, especially if sourced from production leftovers.

Looking beyond alternative materials, the existing plastic litter should not be overlooked, as it continues to accumulate in our environments. Alarmingly, the visible plastic pollution on our beaches and ocean surfaces, though already significant, represents only 5 and 1 %, respectively, of the total plastic litter in the marine environment; the remaining 94 % is found on the ocean floor (Geyer et al., 2017). Although currently shielded from intense degradation and potentially releasing fewer additives than plastics on beaches and ocean surface, the ongoing climate changes pose a significant threat to the balance on these ecosystems and the marine environment as a whole.

A note on the future of plastic research

The general literature on plastic, and plastic leachates, is not at rest, and is instead rapidly expanding, revealing impairments at all levels of organisms. However, laboratory studies are limited to testing the impact of only a few polymers, additives, or POPs, either individually or at best, simultaneously. Yet, in alignment with prior studies, we have underscored the significant dependence of plastic toxicity on multiple factors, such as composition, desorption conditions, and dose. Indeed, even when considering the same leachate solution, the effects on a given behavior can vary radically based on the concentration and desorption conditions of the leachates (see Chapters 4 and 5).

Given the vast array of plastic types, compositions (monomers and additives), potential interactions with contaminants already present in the environment and dependency on abiotic factors, it does not appear to be risky to suggest that accurately understanding the environmental impact of plastic and predicting its evolution is challenging to say the least. In this context, is it finally still worthwhile to study the effects of a myriad of different polymers or additives on all possible marine species? This provocative question primarily aims at raising the issue of the environmental relevance of our results. While I neither have the audacity nor the solution to address this question, it remains, in my humble opinion, worthwhile to focus the research effort on alternatives to conventional plastics that are emerging to determine if they provide a real solution or contribute to another problem.

A note on behavioral studies

While behavior is a crucial parameter connecting individuals to their ecosystems and is relatively sensitive to contamination, it has been inadequately explored in the context of assessing the impact of plastics on the intertidal benthic organisms; see Delaeter et al. (2022) for a review. Is this a mere oversight or is there any other reason? Drawing on my own experience with behavioral studies involving three intertidal species and having observed distinct behaviors in each species, it is clear that studying behavior is not straightforward. First and foremost, its sensitivity can be seen as both an advantage and a drawback. The high sensitivity of behaviors makes it challenging to study because even slight changes in the experimental environment (e.g. a few degrees in temperature, a draft, a shadow, a noise) can induce behavioral changes in the observed individuals. Moreover, the analysis of the observed behavioral patterns can be relatively individual-dependent, especially concerning anxiety-related behaviors in crabs.

However, some models are well-established in the literature, notably in *Danio rerio*, which is extensively studied in ecotoxicology (Holcombe et al., 2013a, 2013b; Kalueff et al., 2013; Maximino et al., 2010b, 2010a). In this thesis work, foraminifera seems to be the organism most likely to become a bio-indicator of plastic leachate contamination among the three studied species. Additionally, its role as a bio-indicator is already well-established in various contexts of contaminations such as trace metals (Armynot du Châtelet et al., 2004), oils spills (Ernst et al., 2006), aquaculture pollution (Pawlowski et al., 2014; Vidović et al., 2014) or urban sewage (Burone et al., 2007). Furthermore, foraminifera offer practical advantage in experimentation. They are easy to sample and the experimental procedures demand less constant handling, compared to crabs and barnacles. These factors make foraminifera a favorable choice, being significantly less time-consuming compared to working with crabs and barnacles. Additionally, the analyses are less likely to be impacted by the above-mentioned observer-dependency issue. It is, however, important to note that in the investigation of crab anxiety behaviors, the parameter sensitive to plastic leachate (i.e. the number of individuals entering a black *vs.* white area when disturbed; see Chapter 3) is not observer-dependent and require much less effort than the analyses of videos needed for the other parameters as it can be obtain immediately. Nevertheless, the automation of video analyses is becoming more common in ecotoxicological studies and would help overcome examiner-dependency issues.

In conclusion, even though the study of behavioral changes may not be advisable or applicable to all species, some, such as foraminifera, remain potential bio-indicators in the study of the impact of plastic leachates. This is particularly significant given the central role of behavior in ecology and the cascading indirect effects of modifications in individual behaviors.

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ANNEXE 1

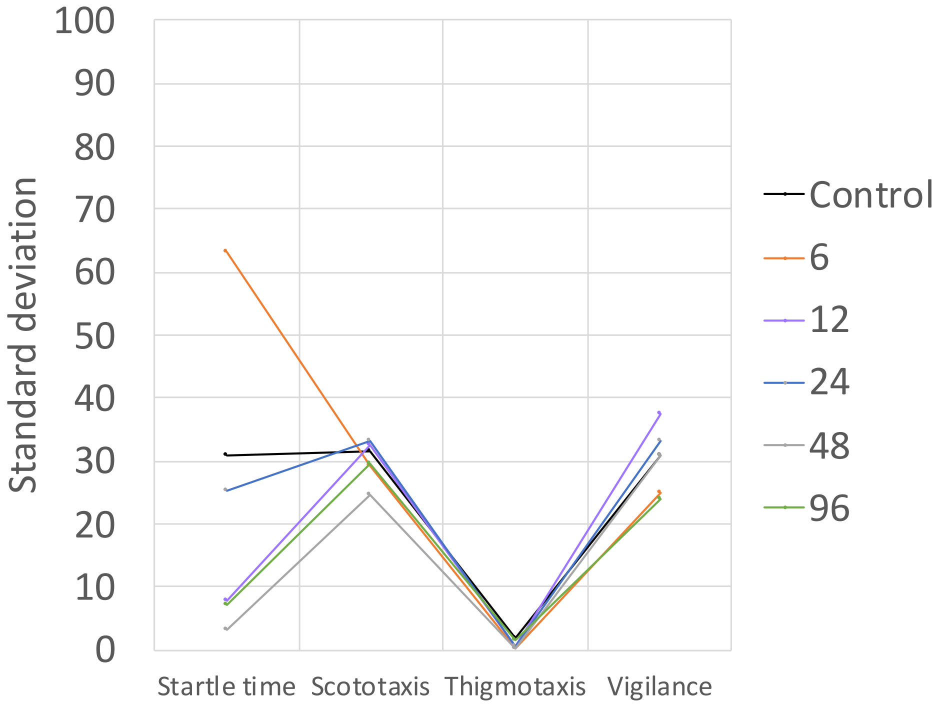
Annexe 1. List of the 57 additives screened by our GC-HRMS analysis.

Function	N°	Molecules	Abbreviation	CAS
Plasticizers	1	Dimethyl phthalate	DMP	131-11-3
	2	Diethyl phthalate	DEP	84-66-2
	3	Di-allyl phthalate	DAlP	131-17-9
	4	Diisobutyl phthalate	DIBP	84-69-5
	5	Di-n-butyl phthalate	DBP	84-74-2
	6	Tributyl Acetyl Citrate	ATBC	77-90-7
	7	Di-n-hexyl phthalate	DHP	84-75-3
	8	Benzyl butyl phthalate	BBP	85-68-7
	9	Bis-2-Ethylhexyl Adipate	DEHA	103-23-1
	10	Diisoheptyl phthalate	DIHP	71888-89-6
	11	Tri(2-ethylhexyl) phosphate	TEHPA	78-42-2
	12	Dicylcohexyl phthalate	DCHP	84-61-7
	13	Bis(2-Ethylhexyl) phthalate	DEHP	117-81-7
	14	Diisononyl hexahydrophthalate	DINCH	166412-78-8
	15	Di-n-octyl phthalate	DIOP	117-84-0
	16	Diisononyl phthalate	DINP	68515-48-0
	17	Di-nonyl phthalate	DNP	84-76-4
	18	Diisodecyl phthalate	DIDP	68515-49-1
Flames retardants	19	Triethyl Phosphate	TEP	78-40-0
	20	Tripropyl Phosphate	TPP	115-86-6
	21	Tributyl Phosphate	TBP	126-73-8
	22	2,4,6-Tribromophenol	2,4,6,TBP	118-79-6
	23	Tris(2-Chloroethyl)Phosphate	TCEP	115-96-8
	24	Tris(2-Chloroisopropyl)Phosphate	TCPP	13674-84-5
	25	2,4,4'-Tribromodiphenyl ether	BDE-28	41318-75-6
	26	Tris(1,3-Dichloro-2-Propyl)Phosphate	TDCPP	13674-87-8
	27	Triphenyl Phosphate	TPhP	513-08-6
	28	2,2',4,4'-Tetrabromodiphenyl ether	BDE-47	5436-43-1
	29	Tricresyl Phosphate	TCP	1330-78-5
	30	Tricresyl Phosphate - isomer	TCrP	78-30-8
	31	2,2',4,4',6-Pentabromodiphenyl ether	BDE-100	60348-60-9
	32	Tri-o-tolyl phosphate	TToP	78-30-8
	33	2,2',4,4',5-Pentabromodiphenyl ether	BDE-99	189084-64-8
	34	2,2',4,4',5,5'-Hexabromodiphenyl ether	BDE-153	68631-49-2
	35	2,2',4,4',5,6'-Hexabromodiphenyl ether	BDE-154	207122-15-4
	36	2,2',3,4,4',5',6-Heptabromodiphenyl ether	BDE-183	207122-16-5
	37	1,2-Bis (2,4,6 Tribromophenoxy) ethane	BTBPE	37853-59-1
Antioxydants	38	6,6'-di-tert-butyl-2,2'-thiodi-p-cresol	Irganox® 1081	90-66-4
	39	Butylated hydroxytoluene	BHT	128-37-0
	40	pentaerythritol tetrakis (3-(3,5-di-t-butyl-4-hydroxyphenyl)propionate	Irganox® 1010	6683-19-8
	41	3,5-di-tert-butyl-4-hydroxyhydrocinnamic acid, octadecyl ester	Irganox® 1076	2082-79-3
	42	6,6'-ditert-butyl-4,4'-thiodin-m-cresol	Lowinox® TBM-6	96-69-5
UV stabiliser	43	2,2-dihydroxy-4,4-dimethoxybenzophenone	Uvinul® 3049	131-54-4
	44	2-t-Butyl-6(5-chloro-2H-benzotriazol-2-yl)-4-methylphenol	UV-326	3896-11-5-
	45	2-(2H-Benzotriazol-2-yl)-4,6-di-tert-pentylphenol	UV-328	25973-55-1
	46	2,4-Di-tert-butyl-6-(5-chloro-2H-benzotriazol-2-yl)phenol	UV-327	3864-99-1
	47	2-hydroxy-4-octyloxybenzophenone	Uvinul 3008	1843-05-6
Antioxidants – plasticizers - stabilizers	48	4-Tert-Octylphenol	4-t-OP	140-66-9
	49	Nonylphenol	NPs	84852-15-3
	50	4-nonylphenol	4-NP	104-40-5
	51	Nonylphenol Monoethoxylate	NP1EO	27986-36-3
	52	Bisphenol F	BPF	620-92-8

	53	4-Nonylphenol Monoethoxylate	4-NP1EO	104-35-8
	54	Bisphenol A	BPA	80-05-7
	55	Bisphenol B	BPB	77-40-7
	56	Nonylphenol diethoxylate	NP2EO	N/A
	57	Bisphenol S	BPS	80-09-1

ANNEXE 2

(a)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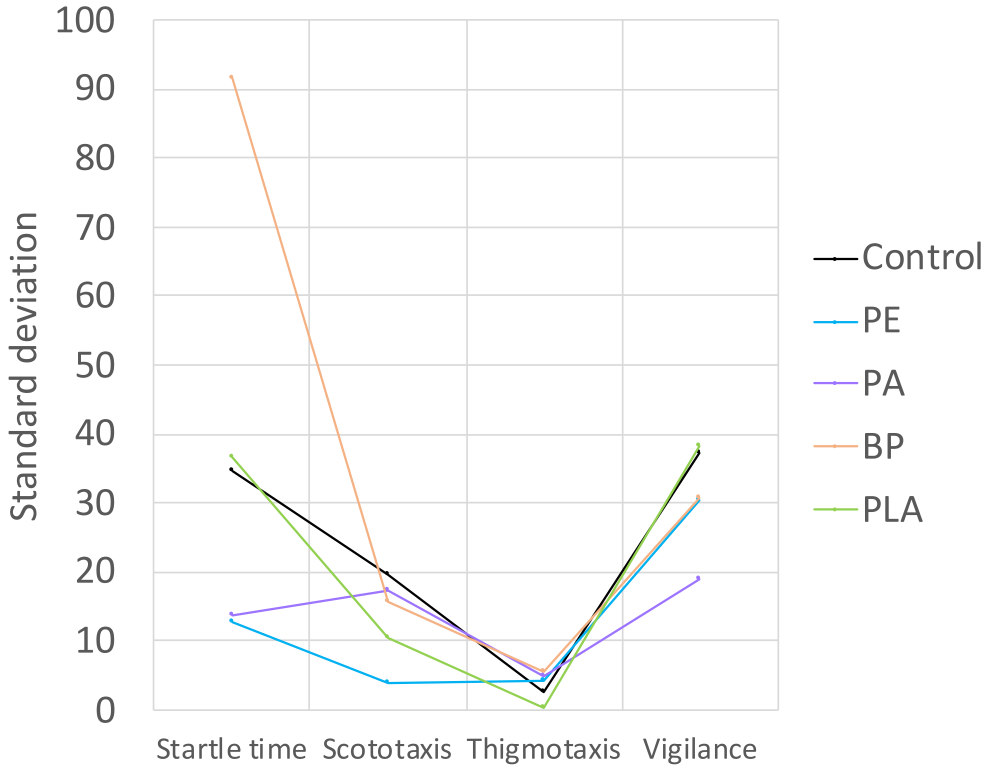
100
90
80
70
60
50
40
30
20
10
0
Startle time
Scototaxis
Thigmotaxis
Vigilance
Control
6
12
24
48
96

(b)


Standard deviation
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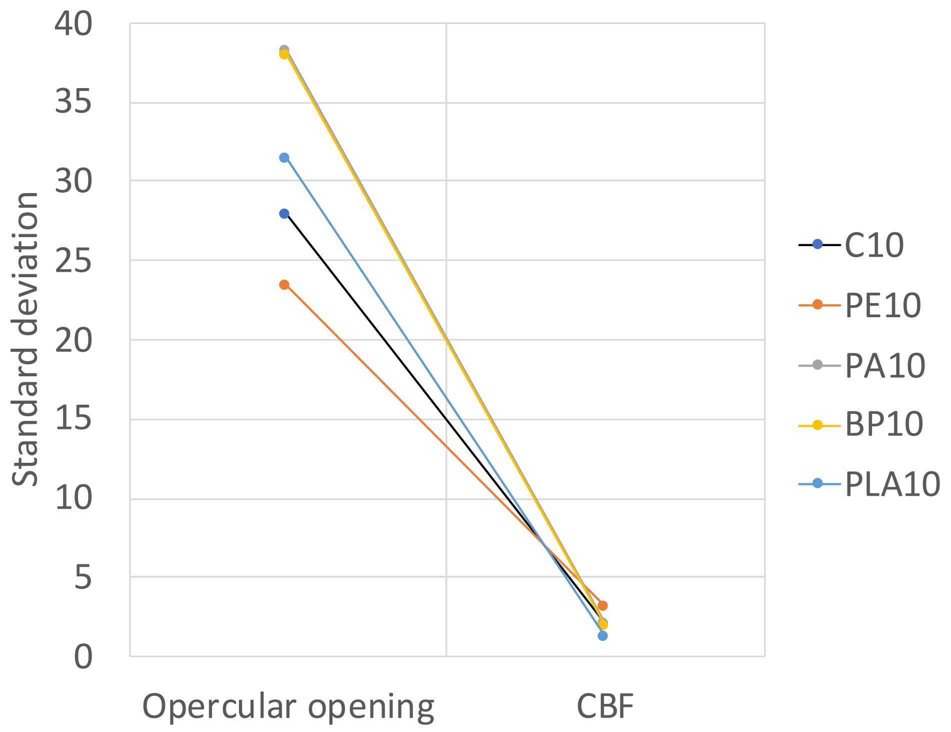
Startle time
Scototaxis
Thigmotaxis
Vigilance
Control
PE
PA
BP
PLA

(c)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35
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10
5
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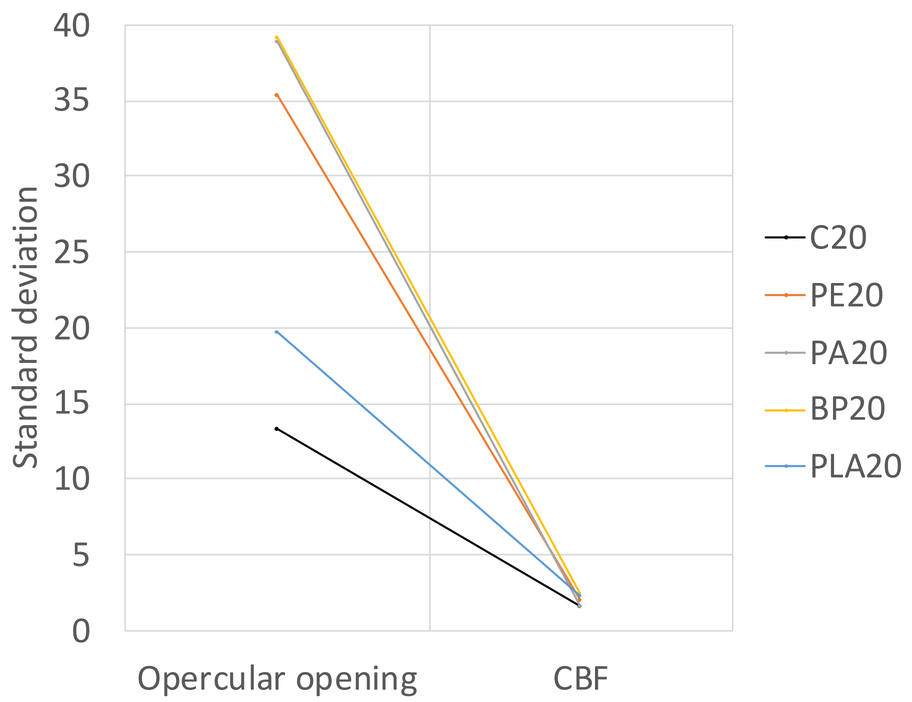
Standard deviation
Opercular opening
CBF
C10
PE10
PA10
BP10
PLA10

(d)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Standard deviation
Opercular opening
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C20
PE20
PA20
BP20
PLA20

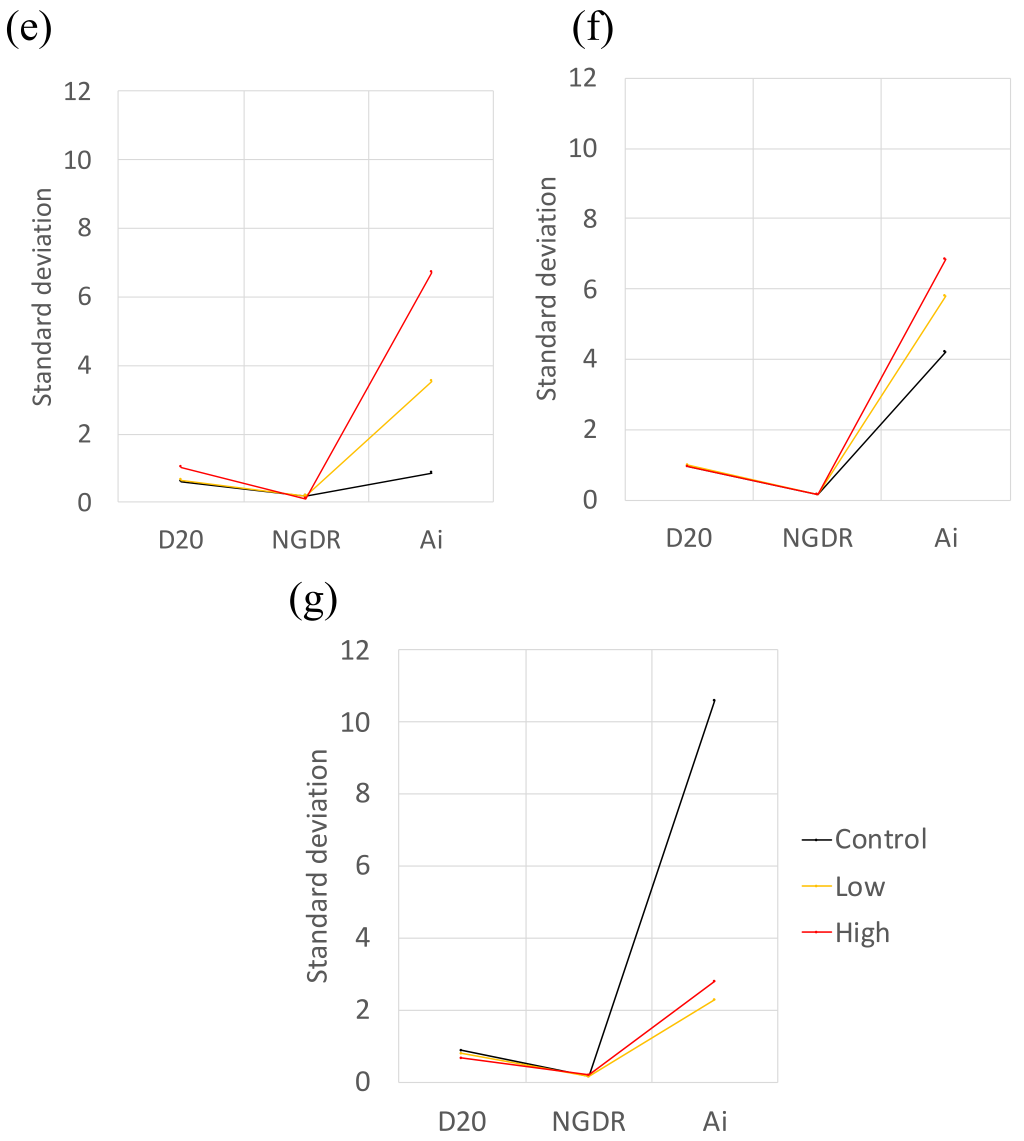

Figure 1. Standard deviations of (a) the impact of mask surgical leachates on the crab *Hemigrapsus sanguineus* depending on the incubation time, the impact of plastic leachates on (b) *H. sanguineus*, (c) the barnacle *Austrominius modestus* for pellets incubated at 10°C and (d) 20°C, (e) the foraminifera *Haynesina germanica* exposed to PE leachates, (f) PA leachates and (g) BP leachates.

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Review

Plastic leachates: Bridging the gap between a conspicuous pollution and its pernicious effects on marine life

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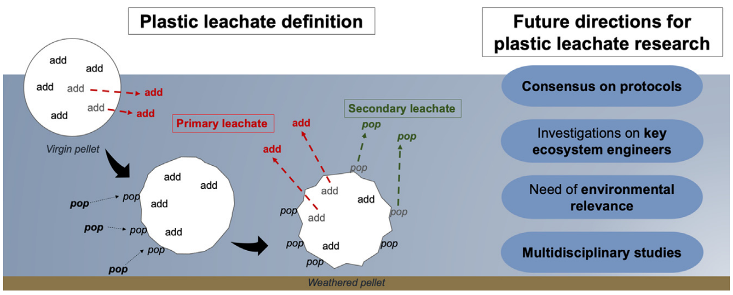
HIGHLIGHTS

- The definition of plastic leachates is refined as primary (additives) and secondary (accumulated contaminants) leachates.
- Plastic leachate literature needs consensus on protocols.
- The impacts of plastic leachates have to be investigated on key ecosystem engineers.
- Plastic leachate experiments lack of environmental relevance.
- Multidisciplinary studies have to be considered for future plastic leachates research.

GRAPHICAL ABSTRACT



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Marine microbes
Marine algae

ABSTRACT

With 4 to 12 million tons of plastic entering the marine environment each year, plastic pollution has become one of the most ubiquitous sources of pollution of the Anthropocene threatening the marine environment. Beyond the conspicuous physical damages, plastics may release a cocktail of harmful chemicals, i.e. monomers, additives and persistent organic pollutants. Although known to be highly toxic, plastic leachates seemingly appear, however, as the "somewhat sickly child" of the plastic pollution literature. We reviewed the only 26 studies investigating the impact of plastic leachates on marine microbes and invertebrates, and concluded that the observed effects essentially depend on the species, polymer type, plastic composition, accumulated contaminants and weathering processes. We identified several gaps that we believe may hamper progress in this emerging area of research and discussed how they could be bridged to further our understanding of the effects of the compounds released by plastic items on marine organisms. We first stress the lack of a consensus on the use of the term '*leachate*', and subsequently introduce the concepts of primary and secondary leachates, based on the intrinisic or extrinsic origin of the products released in bulk seawater. We discuss how methodological inconsistencies and the discrepancy between the polymers used in experiments and their abundance in the environment respectively limit comparison between studies and a comprehensive assessment of the effects leachate may actually have in the ocean. We also discuss how the imbalanced in the variety of both organisms and polymers considered, the mostly unrealistic concentrations used in laboratory experiments, and the lack of

Abbreviations: BBP, Butyl benzyl phthalate; CTR, Car tire rubber; DBP, Dibutyl phtalate; DDT, Dichlorodiphenyltrichloroethane; DEHA, Bis(2-ethylhexyl) adipate; DEHP, Bis(2-ethylhexyl) phthalate; DEP, Diethyl phtalate; DMP, Dimethyl phtalate; HDPE, High density polyethylene; LDPE, Low density polyethylene; PAH, Polycyclic aromatic hydrocarbon; PAM, Polyacrylamide; PAN, Polyacrylonitrile; PBDE, Polybrominated diphenyl ethers; PC, Polycarbonate; PCB, Polychlorinated biphenyls; PE, Polyethylene; PES, Polyester; PET, Polyethylene terephthalate; PFA, Phenol-formaldehyde; PIR, Polyisopropene rubber; PLA, Polyamide; PMMA, Polymethyl methacrylate; POP, Persistent organic pollutant; PP, Polypropylene; PS, Polystyrene; PUR, Polyurethane; PVC, Polyvinyl chloride; SBR, Styrene-butadiene rubber; TWP, Tire wear particles.

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Lack of behavioral effect of surgical mask leachate on the Asian shore crab *Hemigrapsus sanguineus*: Implications for invasion success in polluted coastal waters

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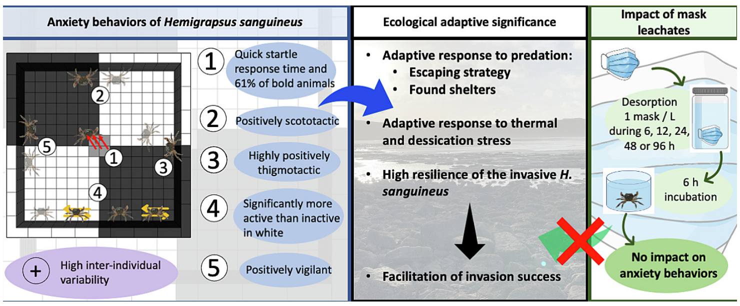
HIGHLIGHTS

- Surgical masks are a new source of plastic mass pollution.
- The effect of mask leachates on marine life is still poorly considered.
- Plastic pollution particularly impact intertidal ecosystems.
- Mask leachates do not impact the behavior of the invasive crab *Hemigrapsus sanguineus*.
- Resilience to contaminant exposure is discussed in an invasion success context in anthropogenically-impacted environments.

GRAPHICAL ABSTRACT



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ABSTRACT

The COVID-19 pandemic generated a new source of plastic mass pollution, i.e. surgical masks, that preferentially accumulate in intertidal environments. Made of polymers, surgical masks are likely to leach additives and impact local intertidal fauna. As typical endpoints of complex developmental and physiological functions, behavioral properties are non-invasive key variables that are particularly studied in ecotoxicological and pharmacological studies, but have, first and foremost, adaptive ecological significance. In an era of ever-growing plastic pollution, this study focused on anxiety behaviors, i.e. startle response, scototaxis (i.e. preference for dark or light areas), thigmotaxis (i.e. preference for moving toward or away from physical barriers), vigilance and level of activity, of the invasive shore crab *Hemigrapsus sanguineus* in response to leachate from surgical masks. We first showed that in the absence of mask leachates *H. sanguineus* is characterized by a short startle time, a positive scototaxis, a strong positive thigmotaxis, and an acute vigilance behavior. Specifically, a significantly higher level of activity was observed in white areas, in contrast to the lack of significant differences observed in black areas. Noticeably, the anxiety behaviors of *H. sanguineus* did not significantly differ after a 6-h exposure to leachate solutions of masks incubated in seawater for 6, 12, 24, 48 and 96 h. In addition, our results were consistently characterized by a high inter-individual variability. This specific feature is discussed as an adaptive behavioral trait, which – through the observed high behavioral flexibility – increases *H. sanguineus* resilience to contaminant exposures and ultimately contribute to its invasion success in anthropogenically-impacted environments.

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Résumé

Le comportement joue un rôle crucial dans la survie des organismes en leur permettant de s'adapter à leur environnement particulièrement variable. De nos jours, les réponses comportementales des organismes aux changements environnementaux doivent faire face à des défis sans précédents en raison des changements rapides et néfastes provoqués par l'ère Anthropique. En particulier, la pollution plastique se distingue comme l'une des préoccupations les plus pressantes dans les habitats marins. Au-delà des dommages physiques évidents, les plastiques peuvent libérer un cocktail nocif de molécules chimiques, compromettant les organismes marins à de nombreux niveaux. Liant les individus au fonctionnement des écosystèmes et aux processus évolutifs, le comportement des organismes reste cependant peu étudié dans la littérature sur l'impact des lixiviats de plastique. Ce travail de thèse vise à combler les lacunes existantes dans la littérature en ce qui concerne les organismes et les polymères étudiés. Après une revue approfondie de la littérature, ce travail se concentre sur l'étude de l'impact des lixiviats de plastique, issus de bio-polymères et de polymères conventionnels sur les comportements liés à l'anxiété chez le crabe *Hemigrapsus sanguineus*, les comportements de déplacement du foraminifère *Haynesina germanica* et les comportements cirraux de la balane *Austromonius modestus*. Les résultats révèlent des modifications significatives de ces comportements, qui dépendent de l'espèce, du type de polymère et de la concentration des lixiviats, et compromettent l'équilibre délicat de l'écosystème. Notamment, le lixiviat de bio-polymère entraine des altérations comportementales similaires, voire plus prononcées, que ceux issus de polymères conventionnels, soulevant des inquiétudes significatives quant à la sécurité environnementale des alternatives aux plastiques.

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Abstract

Behaviors play a pivotal role in organisms' survival, enabling organisms to cope with their ever-changing environment. Nowadays, adaptive behavioral responses to environmental changes face unprecedented challenges due to the rapid and detrimental effects of the Anthropocene era. Noticeably, plastic pollution stands out as one of the most pressing concerns in marine habitats. Beyond causing conspicuous physical damages, plastics may leach a cocktail of harmful chemicals impairing marine organisms at various levels. Despite its role in connecting individuals to ecosystem functioning and evolutionary processes, organism behavior remains scarcely studied in the plastic leachate literature. This PhD thesis aims at to address the gaps in existing literature concerning the organisms and polymers considered. After an extensive review of the plastic leachate literature, this work focuses on investigating the impact of plastic leachates from both bio and conventional polymers on the anxiety-related behaviors of the crab *Hemigrapsus sanguineus*, the motion behaviors of the foraminifera *Haynesina germanica* and the cirral activity of the barnacle *Austrominius modestus*. The results reveal significant modifications in behaviors, highlighting species, polymer and dose dependencies, posing a threat to the delicate ecosystem balance. Noticeably, the biopolymer leachate results in similar or even more behavioral alterations than leachates from conventional polymers, raising significant concerns about the environmental safety of plastic alternatives.