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IMPACT OF POLYAMIDE MICROFIBERS ON MARINE ORGANISMS USING *Mytilus galloprovincialis* AS A MODEL

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Master in Marine and Coastal Systems

Research study performed under the supervision of:

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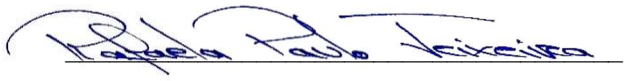
2019

IMPACT OF POLYAMIDE MICROFIBERS ON MARINE ORGANISMS

USING *Mytilus galloprovincialis* AS A MODEL

“Eu, Rafaela Paulo Teixeira, declaro ser a autora da tese cujo título é “Impact of Polyamide Microfibers on Marine Organisms”, que se caracteriza como original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.”

"I, Rafaela Paulo Teixeira declare to be the author of the work whose title is “Impact of Polyamide Microfibers on Marine Organisms”, which is characterizes as original and unpublished. Authors and works consulted are duly cited in the text and are included in the list of references. "



Rafaela Paulo Teixeira

“There is a fascination with the idea that one has ‘seen someone else do something’ before one can achieve it. Maybe that’s true in some cases, but clearly it is not a requirement. I knew what I wanted to do.”

Mae Jemison

Acknowledgments

My journey, in the past two years can't be expressed entirely by words. But the best way to attempt to describe it is by acknowledging the stakeholders that were and will be forever involved in this process:

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"Great things (…) are not done by one person. They're done by a team of people".

Thank you all so much!

Abstract

Plastic is a major source of pollution/contamination in the marine environment, with important impacts on organisms, trophic webs and ecosystems. A major component of these plastic derivatives are polyamide microfibers (PAMF) that originate from synthetic fabrics. Contrary to the so-called microplastics, the impacts of these microfibers on marine species has received little attention. This thesis aims at evaluating the impact of such microfibers on the physiological processes of marine organisms and its possible introduction in the food webs. To do so, *Mytilus galloprovincialis* served as a model, to investigate the physiological impact of PAMF. The quantities of microfibers ingested, accumulation in the digestive tract, effects on growth variables, utilization of energetic substrates and oxidative stress were studied.

The experimental setup included the establishment of a system of 12 tanks distributed by 4 levels of concentration of PAPF in the water (three replicates per treatment level): 0 (Control), 30 (30 PAMF/L), 100 (100 PAMF/L) and 200 (200 PAMF/L). Sixty mussels of similar sizes (length 2-5mm) were placed in each tank and the experiment was run over a 45-day period. At days 15, 30 and 45, 5 specimens from each tank were sacrificed to obtain information on growth (shell biometrics), weight (soft tissues and shell weight), presence of microfibers in the tissues (mantle, gills, digestive glands, oral cavity and byssus) and catalase activity (in digestive glands and gills). Mortality was registered whenever the tanks were monitored (every other day).

Results indicate that the PAMF levels tested do not cause mortality in *M. galloprovincialis* and do not affect shell size. However, significant differences in the weight regarding the shell weight and the dry weight or organic tissue were observed, decreasing with the increase of PAMF concentration in the water. The number of fibers in the different structures was similar (maximum values around 60) with no pattern of retention of the fibers (increase with time or level of PAMF), with the exception of the digestive glands where, for the levels 30, 100 and 200, the retention of fibers increased from day 15 to 30 and then decreased to day 45. Differences in the CAT activity were observed in the digestive glands and gills, in which, overall, the antioxidant activity increased with the increase of PAMF.

In conclusion the polyamide microfibers in the water where *M. galloprovincialis* were grown did not have a highly significant impact of its health along the 45 days of the experiment since it did not cause mortality, likely due to the high capacity of this species to acclimate and adapt. However, future studies will be conducted to better understand the impact of these plastics' derivatives in marine organisms.

Keywords: polyamide microfibers, physiological impact, marine environment, *Mytilus galloprovincialis*.

Resumo

O plástico é uma importante fonte de poluição / contaminação no ambiente marinho, causando grandes impactos a nível dos organismos marinhos, teias tróficas e ecossistemas. Um componente importante desses derivados do plástico são as microfibras de poliamida (MFPA) originárias de tecidos sintéticos. Ao contrário dos chamados microplásticos, os impactos dessas microfibras nas espécies marinhas têm recebido pouca atenção. Esta tese tem como objetivo avaliar o impacto dessas microfibras nos processos fisiológicos de organismos marinhos e sua possível introdução nas redes alimentares. Para isso, *Mytilus galloprovincialis* serviu como modelo para investigar o impacto fisiológico das MFPA. Para isso, foram estudadas as quantidades de microfibras ingeridas, o seu acúmulo no trato digestivo, os seus efeitos nas variáveis de crescimento, o impacto na utilização de substratos energéticos e se estas causam stress oxidativo.

O delineamento experimental incluiu o estabelecimento de um sistema de 12 tanques distribuídos por 4 níveis de concentração de PAPF na água (três replicados por nível de tratamento): 0 (Controle), 30 (30 PAMF / L), 100 (100 PAMF / L) e 200 (200 PAMF / L). Sessenta mexilhões de tamanho idêntico (comprimento 2-5 mm) foram colocados em cada tanque e a experiência foi realizada durante um período de 45 dias. Nos dias 15, 30 e 45, 5 mexilhões de cada tanque foram sacrificadas para obter informações sobre crescimento (biometria da concha), peso (tecidos moles e peso da concha), presença de microfibras nos tecidos (manto, brânquias, glândulas digestivas, cavidade oral e bisso), taxas de respiração e atividade da catalase (glândulas digestivas e brânquias). A mortalidade e a quantidade de partículas fecais produzidas (indicador da quantidade de alimento ingerido) foram registadas sempre que os tanques eram verificados e limpos (a cada dois dias)

Os resultados indicam que as MFPA não causam mortalidade em *M. galloprovincialis* e não afetam seu o crescimento a nível da altura, largura e comprimento da concha. No entanto, foram observadas diferenças significativas ao peso seco da concha e ao peso seco da carne, diminuindo com o aumento da concentração de MFPA nos tanques. O número de fibras nas diferentes estruturas estudadas foi semelhante (valores máximos em torno de 60), incluindo o bisso em que os valores eram altos em um único indivíduo. Não há um padrão claro de retenção das fibras (aumento com o tempo ou o nível de MFPA), com exceção das glândulas digestivas, onde para os níveis 30, 10 e 200 a retenção de fibras aumenta do dia 15 para o 30 e depois diminui para dia 45. Observaram-se diferenças na atividade da enzimática da catalase nas glândulas digestivas e brânquias, nas quais, em geral, a atividade antioxidante aumentou com o aumento dos níveis. A captação de oxigênio apresentou diferentes flutuações ao longo do tempo, diminuindo do dia 15 para o dia 30 (variação de 0 ± 2 mg/L a 0 ± 1 mg/L), diminuindo no dia 45, atingindo níveis mais próximos aos do o dia 15 (a maioria dos valores estão dentro da faixa de 0 ± 2mg/L).

A existência de microfibras de poliamida nos tanques de água de *M. galloprovincialis* não teve um impacto altamente significativo de sua saúde ao longo dos 45 dias da experiência, uma vez que não causou mortalidade devido à alta capacidade de aclimatação e adaptação destes organismos. No entanto, estudos futuros serão realizados para entender melhor o impacto dos derivados desse plástico nos organismos marinhos. No entanto, estudos futuros serão realizados para entender melhor o impacto dos derivados desse plástico nos organismos marinhos, uma vez que não podemos excluir a possibilidade de sua toxicidade, pois estes constituem um dos principais contaminantes / poluentes da atualidade nos ecossistemas marinhos.

Palavras-chave: microfibras de poliamida, impacto fisiológico, ambiente marinho, *Mytilus galloprovincialis*.

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List of Abreviations

AAM	Anterior adductor muscle
AChE	Acetylcholinesterase
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
ARA	Arachidonic acid
CAT	Catalase
CCMAR	Center of Marine Sciences
df	Degrees factor
DG	Digestive glands
DNA	Deoxyribonucleic acid
EC	Extintion Coeficient
EPA	Eicosapentaenoic
G	Gills
GPS	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione s-transferase
LPO	Lipid peroxide
MFPA	Microfibras de Poliamida
MS	Mean of Squares
PAM	Posterior Adductor Muscle
PAMF	Polyamide Microfibers
REP	Replicates
ROS	Reactive Oxygen Species
SOD	Superoxidase dismutase
SS	Sum of Squares
stdev	Standard deviation

1 INTRODUCTION

Plastics are synthetic polymers derived mainly from fossil fuels and due to their high versatility and low production costs, plastics substituted many other traditional materials and are now widespread (Barnes *et al.*, 2009; Thompson *et al.*, 2009; GESAMP, 2015; Cesa *et al.,* 2017). Plastics have a long residence time in the environment, where due to mechanical degradation they may appear in different forms and sizes namely nano, micro, meso and macro scales. Nanoparticles range between 100 nm and 1mm (Lee *et al.*, 2013; Costa *et al.*, 2016), microparticles between 1 and 5 mm (Arthur *et al.,* 2009, Lee *et al.*, 2013, Miller *et al.*, 2017), mesoparticles between 5 and 25 mm and macroparticles are larger than 25mm (Lee *et al.*, 2013).

Plastic is massively produced since the 1950's (UNEP, 2016) and its production has increased to 300 million tonnes in 2014 (Plastic Europe, 2015). In other words, plastic has become indispensable in our daily life. Quoting (Cesa *et al*, 2017), "*this consumption followed a paradigm change: from limited prognostics of a cleaner world* (Derraik, 2002; Thompson *et al.* 2009) *to the necessity of comprehending what is considered one of the main anthropogenic footprints of our age (Barnes et al., 2009)*". Therefore, micro and nanoplastics have been recognized as emerging pollutants (Engler, 2012; Rochman *et al.*, 2014; Wang *et al.*, 2016; Kolandhasamy *et al.,* 2018).

Polyamide microfibers are disposed in marine ecosystems mainly through cloth industry waste (Browne *et al.*, 2011) (Figure 1) and through the use of industrial, commercial or household washing machines. It is estimated that current textiles contain 170% more synthetic fibers (per instance, polyester, acrylic, polypropylene, polyethylene and polyamide fibers) than natural ones (e.g.: cotton, wool, silk, etc.) and a garment can shed more than 1900 fibers per wash (Browne *et al.,* 2011).

1.1. Mytilus galloprovincialis characterization

Molluscs, such as bivalves, are used as indicator organisms for environmental quality assessments since they are generally sessile, live in close contact with benthic substrates and sediment and are filter feeders (Faucet *et al*., 2004; Mulcahy, 2000). Thus, mussels such as *M. galloprovincialis* are sentinel species highly used in toxicology and ecotoxicology (Faucet *et al*., 2004). Some of the most organs used in ecotoxicological studies are the mantle, gills and digestive glands (Le Pennec and Le Pennec, 2001; Faucet *et al*., 2004).

The *Mytilus galloprouincialis* (Mediterranean mussel) is a commercial species It was firstly described by Lamarck (1819) (Table 1) and it occurs from the Mediterranean until the Atlantic coast of Europe (Gardner, 1992). It was also documented the presence of this mussel in northwestern Ireland, southwest England, west coast of North America (McDonald and Koehn 1988) and Australasia (McDonald *et al*., 1991), showing physiological adaptations to warm water environments (Gardner, 1992). In this way, it appears that its geographic distribution is determined primarily by the water temperature (Gardner, 1992).

Table 1 Scientific classification of *Mytilus galloprovincialis* Lamarck, 1819 (WoRMS, 2019).

Scientific Classification	
Kingdom	Animalia
Phyllum	Mollusca
Class	Bivalvia
Subclass	Pteriomorphia
Order	Mytilida
Superfamily	Mytiloidea
Family	Mytilidae
Subfamily	Mytilinae
Genus	*Mytilus*
Species	*Mytilus galloprovincialis*

These mussels feed on phytoplankton and detritus filtered from the water column and theirs dimensions are highly influenced by biotope (intertidal shells usually remain small with an usual maximum of 6 cm, while deep-water shells can easily achieve 9 cm long) (FAO, 2019). These bivalves have a dorsoventral compressed body and bilateral symmetry (Kukenthal *et al*., 1985). It has a tongue like foot capable of expanding, serving as a way for the animal to move and to dig in the substrate (Kukenthal *et al*., 1985). This expansion is due to the pressure of the hemolymph in the interior of the tissue (Kukenthal *et al*., 1985)

The *M. galloprovincialis* can attach in the substrate, including, rocky intertical zones though the byssus (Sagert and Waite, 2009). This structure has hundreds of threads with adjustable tension analogous to tendons (Sagert and Waite, 2009). However, tendons transfer energy while the byssus dissipates it through extendions > 10% (Gosline *et al*., 2002).

The two valves are connected to each other by a hinge responsible for the passive gaping of it (Saphoerster, 2008). The two adductor muscles are the ones responsible to regulate its open, namely the posterior adductor muscle (PAM) and the anterior adductor muscle (AAM) (Saphoerster, 2008) and the force in these muscles can seal the mussel hermetically. *et al*

The gills play an important role in the *M. galloprovincialis* organism and it is more than a respiratory surface (Bierbaum and Shumway, 1988; Jones, Richards and Southern, 1992). The water pumping and the food particles sorting, transport and removal are done by the ciliary mechanisms of the gills (Jones, Richards and Southern, 1992; Morton, 1983; Yonge, 1947). After ingestion and sorting of the organic compounds, the digestion and assimilation of it occur in the digestive glands (Faucet *et al*., 2004).

1.2. Occurrence and impacts

Due to the high use of synthetic clothing and access to mechanical washing, the microfibers' concentration is higher in industrial and urbanized areas (Browne *et al.*, 2011) (Figure 1). The discharges of polyamide from washing machines contaminate the environment at rates of more than 100 fibers per liter of effluent and aquatic organisms can uptake these contaminants (Figure 1). Since polyamide microfibers have similar sizes as plankton, they can be ingested by organisms that feed at this trophic level (Wright *et al.*, 2013; Pirc *et al.,* 2016; Ory *et al.*, 2017; Kolandhasamy *et al.,* 2018) and have the potential to cause serious toxic effects. PAMF

have been found in the stomach and foregut of several organisms (Watts *et al., 2015*) including bivalves, crustaceans, fish, birds and mammals. Depending on the trophic level, their health can be affected through deleterious effects such as gut blockage and false satiation leading to malnutrition or tissue damage (Watts *et al., 2015*). It is as yet unclear if ingested PAMF may evoke other direct toxicological impacts. In addition, PAMF can be ingested by humans through marine organisms' consumption (Figure 1).

Understanding the physiological impacts of the PAMF can help creating awareness and lead to changes in consumer habits with respect to clothes choices. Through consumers' preferences it is possible to induce changes in washing machine manufacture, such as the use of filter bags to trap the fibers and avoid its washing into the environment.

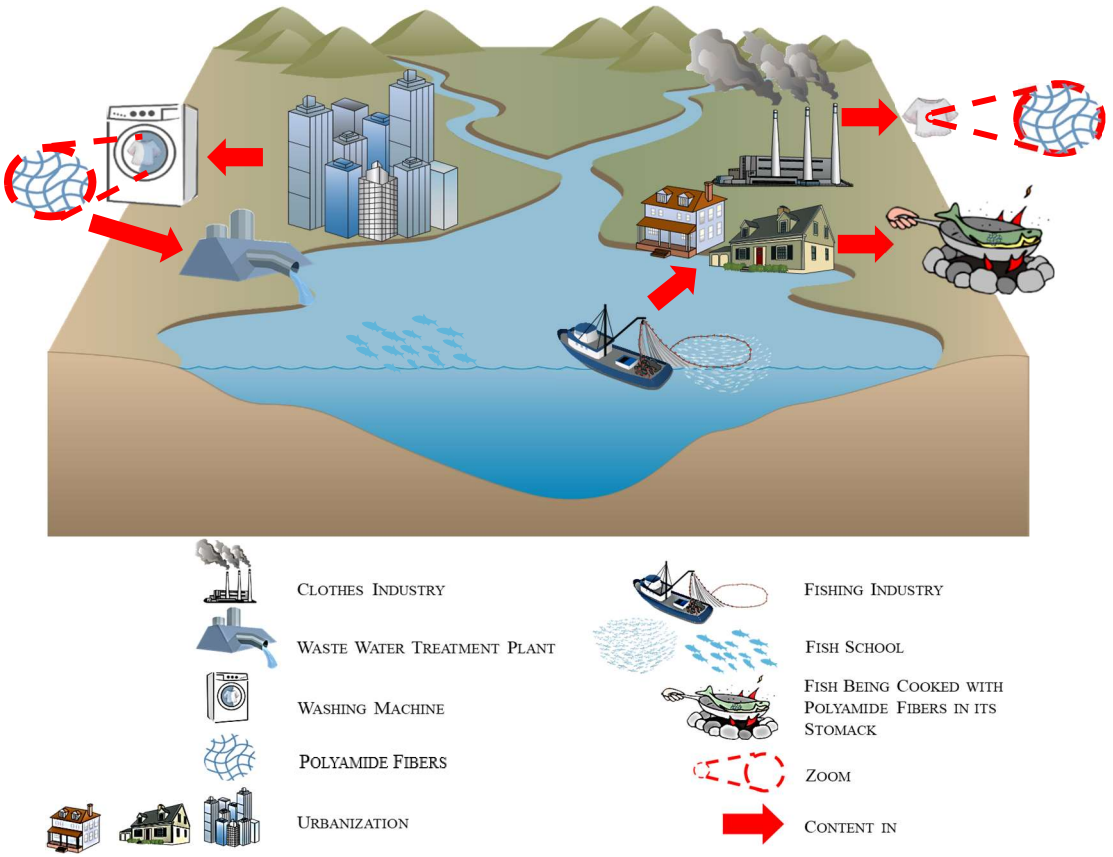


Figure 1 Conceptual model showing the flux of polyamide microfibers in the environment (symbols from Integration and Application Network (ian.umces.edu/symbols)).

The first evidence of plastic in the oceans was in the 1970s (Buchanan, 1971; Carpenter and Smith, 1972), in a time when its production was still very reduced in comparison to current levels (Gago *et al.,* 2018). Since then, the concentration and diversity of plastics has been increasing, becoming a worldwide problem. In fact, recent estimations indicate that approximately eight million metric tons (4.8–12.7 t) of plastic enter the ocean every day (Jambeck *et al.,* 2015).

Previous studies shown that microplastics occur in all continents, in both freshwater and marine environments (Barnes *et al.*, 2009), along beaches (Browne *et al.*, 2011), in sediments (Claessens *et al.*, 2011) and in the water column itself (Eriksen *et al.*, 2013; Foley *et al.*, 2018). In fact, 94% of the microplastics in the Northeast Atlantic Ocean are present in the surface layers (Eriksen *et al.*, 2014; Lusher *et al.*, 2014; Miller *et al.*, 2017). In the surface waters of the Northeast Atlantic Ocean, 95% of all plastic particles present are fibers (Watts *et al,* 2015). They are also present in the benthic shores around the world (Browne *et al.,* 2011) with concentrations of 31 fibers/kg in samples taken from the Cornish estuarine sandy sediments (UK) (Thompson *et al.,* 2004), 213 fibers/kg of dry sediment taken from Island of Norderney (Germany) (Dekiff *et al.,* 2014) and of about 200-800 fibers/kg dry sediment taken from Nova Scotia (Canada) (Mathalon and Hill, 2014). Although the most common microplastics are microfibers (Nel and Froneman, 2015; Altreuter, 2017; Anderson *et al*., 2017; Bagaev *et al*., 2017; Peng *et al*., 2017), these are not currently being managed or regulated, posing a problem potentially greater than microbeads (Altreuter, 2017).

In recent years, the issue has gained some prominence in mainstream media, but the number of scientific publications is relatively low. The mainstream media is starting to talk about derivate plastic microfibers using titles such as "How your clothes are poisoning our oceans and food supply" (Messinger, 2016) but the information to the general public about the harm of polyamide microfilaments is not as widespread as that concerning polyethylene microplastics, likely due to the lack of scientific studies. In fact, a quick search at the "Science Direct" webpage (Elsevier, 2018) with the keywords "Polyamide microfibers" shows just 26 published papers related to marine and environmental pollution from 2005 until 2018. However, none is specifically related to polyamide fibers.

The first author to recognize the link between microfibers in the marine environment and wastewater from clothes washing machines was Browne *et al.* (2011). In his study he took samples from sediments from sandy beaches around the world (Port Douglas and Busselton Beach in Australia, Kyus in Japan, Dubai, Vina Del Mar and Punta Arenas in Chile, Malapascua Island in the Philippines, Faro and Ponta Delgada in Portugal, Virginia and California in the United States of America, Western Cape in South Africa, Pemba in Mozambique and and Sennon Cove in the United Kingdom). It was concluded that the main plastic derivatives released to the environment were fibers, including washing machines released fibers. Following studies indicated that there are toxic effects of plastic derived microfibers (PM) on marine organisms (Watts *et al.,* 2015; Pirc *et al.,* 2016; Taylor *et al.,* 2016; Ribeiro *et al.,* 2017). These include negative impact on feeding efficiency (Wegner *et al*., 2012; Ogonowski *et al*., 2016), growth (Au *et al*., 2015; Jeong *et al*.,2016), reproductive capability (Della Torre *et al*., 2014; Ogonowski et al., 2016) and survival (Booth *et al*., 2016; Luís *et al*., 2015). Most of the negative effects are attributed to the blocking of the feeding structures by these materials (Wright *et al*., 2013b, Eerkes-Medrano *et al*., 2015) and by competing with real food reducing its consumption (Foley, Malinich and Höök, 2018). Despite these adverse effects, there is some evidence showing that organisms can avoid the consumption of these pollutants through either active or passive feeding selection strategies, although not all organisms are capable of doing so (Table 2).

Table 2 Passive and active feeding selection strategies of *Calanus helgolandicus* and *Acartia clausi* copepods, *Tripneustes gratilla* sea urchin larvae, *Eurytemora affinis* copepods and *Holothurian* sea cucumbers in the presence of microplastics' derivatives (adapted from Foley, Malinich and Höök, 2018).

Organisms	Passive and Active feeding selection strategies	Scientific sources
Calanus helgolandicus and *Acartia clausi* copepods	The organisms selected smaller preys rather than the microplastics	Cole *et al.*, 2015; Donaghay and Small, 1979
Tripneustes gratilla seaurchin larvae	The organisms selected microalgae instead of polyethylene beads in presence of food	Kaposi *et al.*, 2014
Eurytemora affinis copepods	The organisms selected latex beads at a higher rate over diatoms prey	Powell and Berry, 1990
Holothurian sea cucumbers	The organisms selected nylon and polyvinyl chloride fragments over sand, since it was easier to ingest	Graham and Thompson, 2009

Microplastic derivatives can adhere to natural prey, such as seaweed and fish eggs (Kashiwada, 2006; Gutow *et al*., 2016) and can adhere to the gills (Kashiwada, 2006; Watts *et al*., 2014). Once ingested, these pollutants can be adhered to the gut walls (Browne *et al*., 2008; Snell and Hicks, 2011) and remain in the digestive tracts for periods of weeks before excretion (dos Santos and Jobling, 1991; Browne *et al*., 2008; Cedervall *et al*., 2012; Batel *et al*., 2016) moving through the trophic web (Murray and Cowie, 2011; Farrell and Nelson, 2013) and being transported to adjacent ecosystems and new geographic locations (Clark *et al*., 2016).

In general, polyamide microfibers have been identified in the digestive tracks of different organisms. For instance, in the digestive tracks of commercial fish from the Adriatic Sea (Figure 2), such as *Sardina pilchardus* (European pilchard/sardine, Fishbase), *Squalus acanthias* (picked dogfish, Fishbase), *Merluccius merluccius* (Senegalese hake, Fishbase), *Mullus barbatus* (surmullet, Fishbase) and *Cheldonicthys lucernus* (gurnard, Fishbase) (Avio, Gorbi and Regoli, 2015). From all the plastics observed, 4% were Nylon (polyamide) (Figure 2) (Avio, Gorbi and Regoli, 2015).

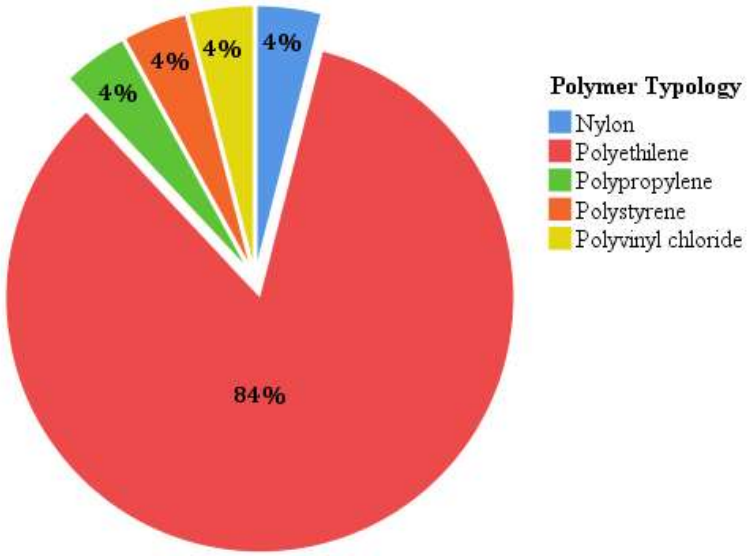


Figure 2 Types of microplastics polymers extracted in Adriatic fish species (adapted from Avio, Gorbi and Regoli, 2015).

Watts *et al.* (2015) studied the food consumption and energy balance effects due to plastic microfilaments in the green crab *Carcinus maenas* and they concluded the metabolic costs of oxygen consumption, fecal pellet production and ammonia excretion in exposed animals outweighed the energy obtained through food intake (Watts *et al.,* 2015). A comparison of the green crab with the planktonic copepod *Calanus helgolandicus* and the marine lugworm *Arenicola marina*, in terms of the consequences of feeding on microplastics (Table 3), indicates the response is not consistent across studies (Foley, Malinich and Höök, 2018) since some organisms can be resilient to stress induced by these contaminants (Nasser and Lynch, 2016; Watts *et al*., 2016). The ingestion of microplastic derivatives and its effects can be minimized through egestion (Foley, Malinich and Höök, 2018) and be influenced by the shape of the particles (spheres versus fibers) (Foley, Malinich and Höök, 2018).

Studies using polyamide microfibers on marine animals using *M. galloprovincialis* were not performed until this day. However, some studies have shown the impact of plastics derivatives in bivalves and mussels.

De Witte et al. (2014) detected microscopic synthetic fibers with a range of 200 to 1500 µm in the soft tissues of wild and commercial mussels samples (Mytilus edulis, Mytilus galloprovincialis and M. edulis/galloprovincialis hybrid form) from the Belgian coasts (three groynes and three quayside locations) and three Belgian supermarkets (Table 4). In the North Sea there was detected an average of 0.36 and 0.47 particles/g in M. edulis and the Pacific oysters C. gigas. However, after a 3-day depuration period, the amount of particles decreased to 0.24 and 0.35 particles/g in mussels and oysters, respectively (Van Cauwenberghe and Janssen, 2014) (*Table 3*). Afterwards, a study published by Li et al. (2015), showed that higher amounts of microplastics were found in nine species of Chinese commercial bivalves, with concentrations between 2.1–10.5 particles/g (*Table 4*).

Mussels (*M. edulis*) collected at six locations along the French–Belgian–Dutch coastline were submitted to a 24 h-clearance completely empty the gut. It was observed an average 0.2 _ 0.3 particles/g (size range 20–90 µm), with a maximum value of 1.1 particles/g (Van Cauwenberghe *et al*., 2015) (Table 4).

Table 3 Comparison between three marine invertebrate species: the planktonic copepod *Calanus helgolandicus,* the marine lugworm *Arenicola marina* and the crab *Carcinus maenas* in terms of consequences of feeding on microplastics (adapted from Watts *et al,* 2015).

Variables	***C. helgolandicus***	**Source**	***A. marina***	**Source**	***C. maenas***	**Sources**
Individual weight (g)	$4.522x10^{-4}$		15		27	Toulmond and Tchernigovtzeff, 1984
Food consumption (% body weight per day)	28 - 85	Roman, 1977	0.099	Casado-Martinez *et al.,* 2010	0.00238	Mente, 2003
Gowth rate (% body weight per day)			0.133 (0.66% food ration)	Casado-Martinez *et al.,* 2010	0.07 (1% food ration)	Barnes *et al.,* 2009
Starvation tolerance (days)	21-Mar	Borchers and Hutchings, 1986			60 – 90	Mente, 2003
Oxygen consumption (mg O_2 g per day)	6.91	Dagg, 1977	1.129	Dagg, 1977	0.921	Dagg, 1977
Effect of ingested plastic	2 – fold reduction in energy stores, reduction in egg hatching success		Energy reserves depleted by up to 50% over 4 weeks		Slight but significant reduction in scope of growth	Wright, Thompson and Galloway, 2013

Table 4 Occurrence of microplastics in bivalves, its average content, method of analysis and respective reference (adapted from Petersen, 2016).

Bivalves	Microplastic average content (SD)	Method of analysis	Reference
Mytilus edulis, commercial mussels, from three Belgian supermarkets. Wild mussels, from Belgian groynes (three locations) and quaysides (three locations)	0.37 (0.22)(a) particles/g wet weight (n = 9) Size: 200–1,500 µm	Extraction/digestion with $HNO_3/HClO_4$, detection counting microscope, confirmation with hot point test Method blanks used	De Witte *et al*., 2014
Commercial bivalves: *Mytilus edulis*, from one location (mussel farm), *Crassostrea gigas*, from one location (supermarket)	*M. edulis*: 0.36 (0.07) particles/g wet weight (n = 72) *C. gigas*: 0.47 (0.16) particles/g wet weight (n = 21) Size: 5–25 µm (55–100%), > 25 µm (0–45%)	Extraction/digestion with HNO_3, detection/counting microscope, confirmation (subset) with Raman Method blanks used	Van Cauwenberghe and Janssen , 2014
Commercial bivalves (9 species), from a fish market in China	Median 4.0, range 2.1–10.5 particles/g (n = 9) Size: 5–250 µm (60%), 5–5,000 µm (40%)	Extraction/digestion with H_2O_2, floatation with NaCl, filtered over 5 µm, detection/counting microscope, confirmation (subset) with µ-FT-IR Method blanks used	Li *et al*, 2015
Mytilus edulis, French-Belgian-Dutch coastline, six locations	0.2 _ 0.3 particles/g (size range 20–90 µm) Size: 20–90 µm	Extraction/digestion with HNO_3, detection/counting microscope, confirmation (subset) with Raman Method blanks not indicated	Van Cauwenberghe *et al*., 2015

The ingested microplastics' derivatives can translocate from the gut to other body parts (Browne, 2008). Laboratory trials involving mussels (*M. edulis*) have shown that ingested polystyrene microspheres of 3.0 or 9.6 µm can translocate from the gut cavity to the hemolymph within 3 d (Browne, Galloway and Thompson, 2008). Given this, it is important to understand the impact of these derivatives in order to formulate future action, rules and legislations for each particular chemical type of derivatives.

1.3 Oxidative Stress

Oxidative stress is "*an expression used to describe various deleterious processes resulting from an imbalance between the excessive formation of Reactive oxygen species (ROS) and limited antioxidant defences"* (Turrens, 2003).

ROS describe a variety of molecules and free radicals that are chemical species with just one unpaired electron derived from molecular oxygen (Turrens, 2003). The oxygen consumed by the organisms is reduced to water together with oxidation of food intake and energy production. The presence of contaminants or xenobiotics can increase the continuous production of ROS and act as "unwanted bi-products" of the biotransformation from endogenous processes, including self-oxidation of heme proteins and electrons nuclear transport (de Zwart *et al.*, 1999; Livingstone, 2001).

Fluctuations in the steady-state concentration of oxidants can make changes in the intracellular signaling (Droge, 2002), uncontrolled increase concentrations of the oxidants leading to free radical-mediated chain reactions that may target proteins (Stadtman and Levine, 2000), lipids (Rubbo *et al.* 1994), polysaccharides (Kaur and Halliwell, 1994) and DNA (Richter *et al.* 1988; LeDoux *et al.* 1999; Turrens, 2003). However, ROS are produced naturally in the cellular metabolism of aerobic organisms, such as anion superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), peroxyl radical, hidroperoxil radical and hypochlorous acid (Halliwell and Gutteridge, 1999; Kappus, 1987; Valavanidis *et al.*, 2006). These ROS play an essencial roll in the physiological control of cell functions (Halliwell and Gutteridge, 1999).

In this way, PAMF can act as a stressor agent in marine organisms (Browne *et al.*, 2013; Galloway and Lewis, 2016; Luís *et al.*, 2015; Oliveira *et al.*, 2012; Oliveira *et al.*, 2013) and possibly cause oxidative stress in *Mytilus galloprovincialis* if there's an effect in the regulation in the balance of the production of ROS and the detoxification of biological systems (Valavanidis *et al.*, 2006).

In order to prevent and eliminate and repair the effects of ROS, organisms have developed antioxidant defense mechanisms, so that they can prevent oxidative damage, including in the presence of contaminants/pollutants, being an indicator of toxicity (Livingstone, 2001).

1.3.1 Biomarker Catalase (CAT): antioxidant enzime

The production of ROS needs to be equilibrated by enzymatic and non-enzimatic antioxidant defences, that in case of aerobic organisms, they are capable to neutralize ROS (Fenech and Ferguson, 2001). An example of these antioxidant enzymes is the catalase.

Catalase (CAT) is present in the peroxisomes - "small, membrane-enclosed organelles containing enzymes involved in a variety of metabolic reactions" (Cooper Hausman and

Hausman, 2000) and it is responsible for the decomposition of the peroxide into water and oxygen (Halliwell and Gutteridge, 1999).

Antioxidant enzyme biomarkers such as CAT have been used to study the effects of contaminats/pollutants in the environment (e.g. Bebianno et al., 2014, Bocchetti *et al.*, 2008, Silva *et al.*, 2012, Vlahogianni *et al.,*2007). In this study, CAT is used as an antioxidant enzyme biomarker to study if the PAMF cause oxidative stress in *Mytilus galloprovincialis*.

1.4. Objectives

For this study to be the most effective in terms of material resources and available time, it was necessary to limit the scope and nature of the problems defined in the thesis plan. Initially it was proposed the study would comprehend two parts related to two different levels of the effect of microfibers on marine organisms. The first level included the effects of microfibers in the water on a filter feeder, the mussel *M. galloprovincialis*. Mussels subjected to different levels of contamination would then be used on a second set of experiments where they would be fed to a carnivorous fish, the seabream (*Sparus aurata*). Although the planed work will proceed (the mussels were kept frozen for later work), this thesis includes only the first phase of experiments, namely, the study of microfibers in the water on *M. galloprovincialis*.

The main goal of this research is to study the potential toxicity of polyamide microfibers (PAMF) on marine organisms, using the species *M. galloprovincialis* as a model, by exposing specimens in sea water containing different concentrations of microfibers. Several aspects and potential effects were considered: entry and accumulation rate of the fibers, impact on specific growth rates and physiological responses such as oxidative stress. We hope to contribute to filling the existent gaps in knowledge and relate with the impacts of other plastic derivatives.

The specific main questions of this study are:

- Do PAMF have an impact on the digestive ability of the *Mytilus galloprovincialis*?
- Do PAMF affect its growth?
- Do PAMF cause oxidative damage along time?
- Do PAMF cause mortality?

2 MATERIALS AND METHODS

The experimental phase of the study was conducted during 7 months. It includes an experimental set up, preliminary tests, animal and data collection, analysis and report drafting.

1.1 Preparation of the Polyamide Fibers

Polyamide sewing fibers were obtained from commercial sources. Sewing one-stranded transparent 100% polyamide thread 0.2 mm thick was used (COATS CRAFTS). Micro fragments were prepared by cutting the thread in sizes between 150 µm and 1000 µm using scissors.

A fixed number of fragments (polyamide microfibers, PAMF) was weighted, containing a mixture of the different lengths. To convert the PAMF concentration to weight, six samples of 50 microfibers were obtained (counted using a magnifying glass) and weighted using an electronic microbalance (SARTORIUS MSP). The mean weight of the six samples was calculated (mean = 0.564 mg, sd = 0.115). From this point onwards, this reference mean weight was used to obtain different concentrations of PAMF

2.2. Model Organism

The model organism selected for this study was the Mediterranean mussel, *M. galloprovincialis*. This species was chosen due to the fact that it is a filter feeder, thus likely to be affected by debris or other particulate elements in the water column. Mussels are also at a lower trophic level being part of several trophic chains (Sarà, Zenome and Tomasello, 2009). Additionally, they are a local species (regional relevance) and easily available. Finally, mussels are frequently used in environmental, ecotoxicology studies and as indicators of pollution/contaminants (Amiard *et al.,* 1986) that provided a large body of information about the species features and cultivation, relevant for the planning of the experiment and comparison of results (Cantillo, 1998).

All the procedures involving the model organisms were done according to the Code of Conduct for Responsible Fisheries (CCRF) principles in the Mediterranean Region that has been recognized by the Federation of European Aquaculture Producers (FEAP) (FAO, 2018).

2.3.Animal Collection

Field campaigns were conducted to catch a total of 750 *M. galloprovincialis* with shell length ranging between 2 and 4.5 cm. The collection site was the pillars of the Praia de Faro Bridge (Faro, Portugal) (Figure 3A and Figure 3B), located in the Ria Formosa Lagoon with the coordinates 37.008318, -7.994535 (Figure 3C). The specimen's collection happened during March 2019. The adequate permits were secured from the competent authorities.

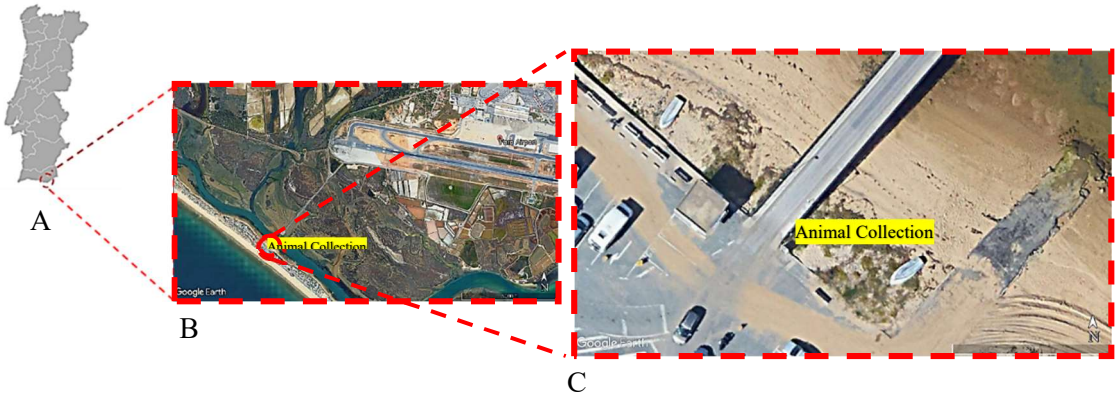


Figure 3 Ria Formosa Lagoon (B) with the coordinates 37.008318, -7.994535 (C) (Faro, Portugal - A) map (Google Earth Pro, 2018).

Four field campaigns were made due to the relatively high number of individuals necessary and they were done in function of the environmental conditions and tides at the sampling site.

During each campaign the mussels were transported in a bucket containing sea water from the Ria Formosa Lagoon and brought to the CCMAR (Center of Marine Sciences – University of Algarve) experimental station, the Ramalhete Station, situated in the Ria Formosa Lagoon. They were further placed in shallow tanks with water flowing directly from the Ria Formosa Lagoon and fed with the lyophilized microalgae *Tetraselmis chuii* (22.1 mg/tank/48 hours)

The mussels were marked individually on their shells using nail polish. Unfortunately, the marks disappeared in days, preventing the collection of repeated measurements on the same specimen. In addition to the loss of individual marks, the specimens formed clusters making it impossible to identify the individuals. Consequently, the biomass was estimated along the study by adding the weight of all clusters in each tank.

2.4. Tank Setup

The experimental tanks were plastic boxes (approximate size 52 x 37 x 16 cm) filled with 30L of seawater and with a rigid mesh tray suspended inside, 15 cm from the bottom of the tank, holding the mussels. The mesh facilitated the attachment of the mussels and the suspension system allowed for easy maintenance operations and warranted a good water flow all around the mussels (Figure 4).

Each tank had 2 compressed air supply tubes, connected to the main air lines of the station and ending in air diffusor stones, placed at opposite corners of the tank. These assured a circular bottom to surface water circulation, spiraling upwards, necessary to prevent the fibers from accumulating at the surface or on the bottom.

The tanks were under a shading cover to keep light exposure as identical as possible (relevant for water temperature and primary production).

Figure 4 Tanks setup constructed with a white box (A), a suspended rigid mesh tray (B), hold in place by plastic tubes (C) knotted with pieces of flexible air tubes (D), air supply tubes (E) coming from the station air supply system (F). A shading cover (G) and side panels of the same material, guaranteed similar sun exposure to all the tanks. The treatment levels and replicates are identified in blue: Level. Each tank had 30L of seawater and sixty *Mytilus galloprovincialis*.

2.5. Experimental Design

All mussels from each experimental condition (12 tanks) were fed with the same concentration of the commercially available dry ready-to-use microalgae *Tetraselmis sp.* with size 4-8 µm, produced by NECTON (Olhão, Portugal). This food supply does not have additives or preservatives and the general composition in dry weight (%) is: protein 42 – 57; lipids 7 – 12; carbohydrate 5 – 12; fiber 0 – 1; it is rich in omega–3 fatty acid (EPA, ARA, ALA), vitamins, aminoacids, minerals and carotenoids. The concentration of dry microalgae given to the organisms was 22.1 mg per tank every 48 hours, immediately after each water renewal. The dry food concentration calculations were done according to the size of the microalgae and the biomass of mussels. A microalgae cell of approximately 4 µm has a mass of 7 pg (Handá *et al,* 2013). According to Wong and Levinton, 2004 the required amount of food is ~3.1 x 10^6 cell/L twice a day. This value was used to calculate the dietary needs for a 48-hour period (Figure 5).

Four levels of PAMF concentration were used: control (0 fibers/L), 30 (30 fibers/L), 100 (100 fibers/L) and 200 (200 fibers/L) with 3 replicates each, making a total of 12 tanks with 720 individuals (60 individuals/tank) (Figure 5).

The trial was conducted for a period of 45 days. The fecal pellet production, biomass and mortality were measured at each water change. All clusters were measured/weighed every 15 days (days 0, 15, 30 and 45). From each tank, five or four mussels were used for the study, thus n = 12 - 15 per condition (Figure 5).

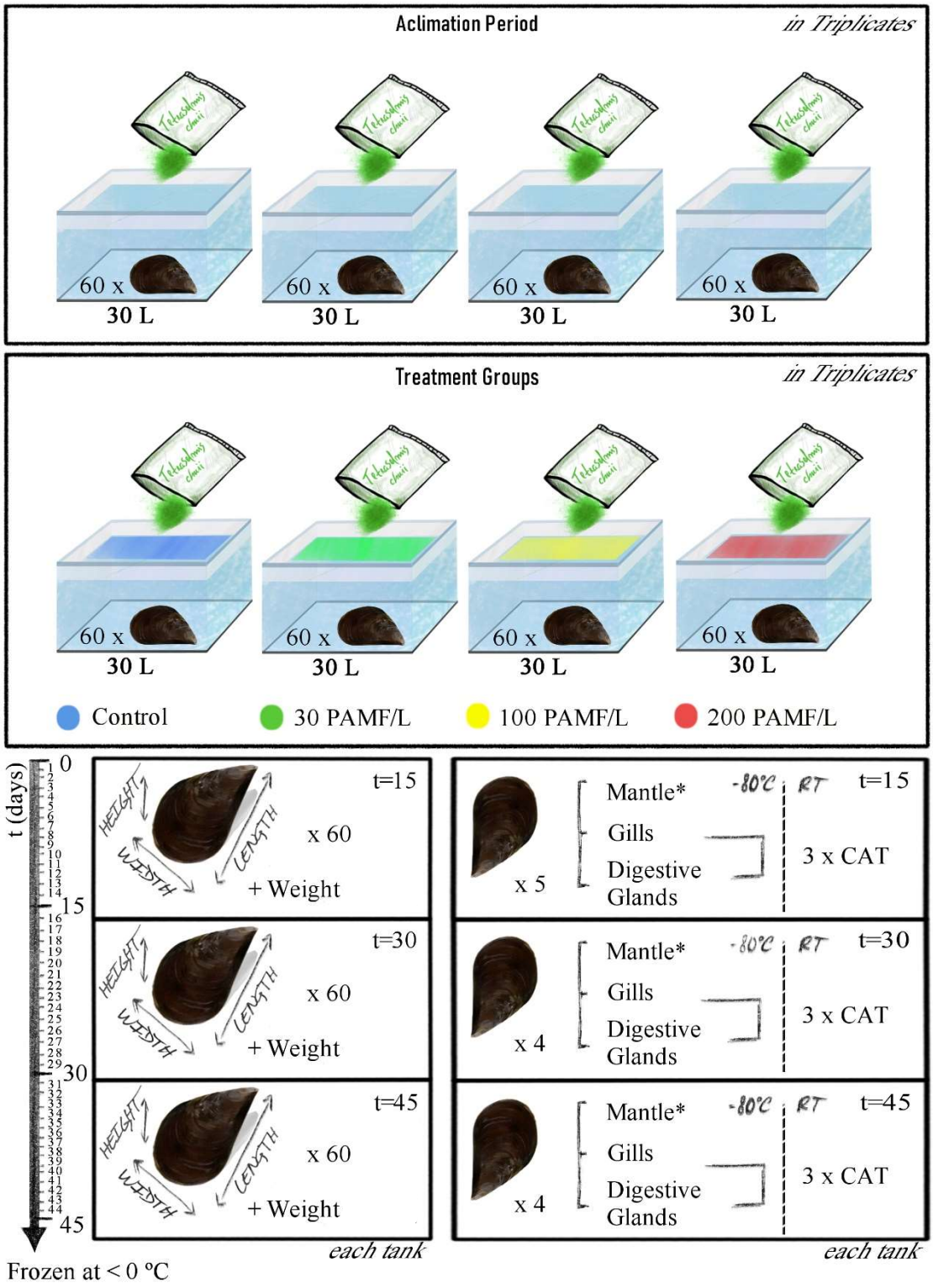


Figure 5 *Representation of the physiological response of polyamide ingestion by the model organisms (experimental design) in which t is the time in days, PAMF is the Polyamide Microfibers and CAT that represents Catalase Activity readings (laboratory analysis performed at each 15 days together with metric measurements (length, width and height) and weight measurements for each treatment. Design tools from KRITA - https://krita.org/en/.*

2.6. System Control and Maintenance

The tanks were initially setup with batches of mussels that were later discarded, in order to verify the stability of the system in terms of water properties such as pH, temperature (ºC), salinity and O_2 (%).

The tanks were filled with seawater pumped from Ria Formosa and the water was renewed every 48 hours. The water quality parameters, namely the temperature (ºC), pH, salinity, conductivity, O_2 concentration (mg/L), O_2 saturation (%) were measured before and after the water change.

In order to maintain the concentration of PAMF in the tanks, each time the water was changed, the tank was cleaned and the adequate amounts of new PAMF were added. During water replacement and tank cleaning the water was filtered through a 150 µm sieve to keep the PAMF and to avoid environmental contamination/pollution.

2.7. Biological parameters

2.7.1. Growth

The height, width, length and lip thickness were measured for each mussel using a digital caliper, the lip thickness was measured 1 mm from the posterior end of the mussel (Figure 6).

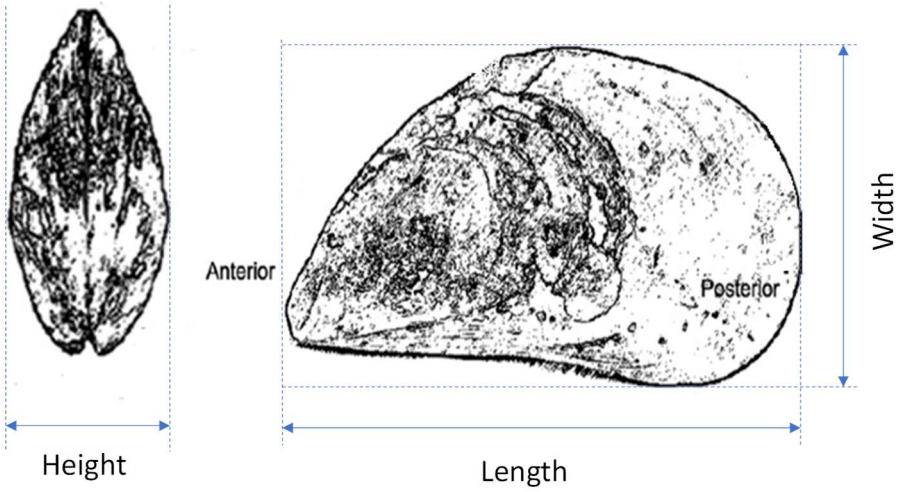


Figure 6 Scheme showing the measurements taken using calliper, with identification of the anterior and posterior shell. A - height; B – length and width (adapted from Auker, 2010).

Five individuals at day 0 and 15 and four individuals at day 30 and 45 were removed for shell measures and individual dry weight.

2.7.2. Survival

Daily monitoring of the tanks and organisms was performed visually. Dead animals were collected and removed with minimal disturbance to the remaining living mussels.

2.7.3. Ingestion of PAMF

Fecal pellets were collected every two days with the use of a plastic pipette and by filtering the water removed from the tanks with a 80 µm filter sieve and gently rinsed three times with distilled water (DI) to remove salts adsorbed to the fecal pellets. Fecal pellets were dried for (24h at 60ºC) in an oven and the dry weight obtained.

2.7.4. Accumulation of PAMF in selected tissues

Five or four individuals per tank were observed at days 15 and four individuals per tank at days 30 and 45.

To investigate the possible presence of PAMF in the tissues, the specimens were dissected and the fibers were counted in several structures: mantle, surface of the gills, digestive glands, oral cavity and byssus.

At 15, 30 and 45 days of the experiment, 4 organisms per tank were sacrificed and dissected in the laboratory to access the contents of the digestive gland (stomach). The amount of PAMF ingested by each organism was determined by counting the fibers under a stereoscope. The presence of PAMF on the surface of the gills, mantle, byssus and around the surface of the oral cavity was also assessed.

The gills, mantle and digestive glands were placed in 1.5 mL microtube and frozen in dry ice immediately after the extraction and transported between the Ramalhete Station and the University of Algarve facility where they were stored in an ultrafreezer at -80ºC until posterior enzymatic or biochemical analysis.

2.8. Oxidative Stress

To evaluate the possible oxidative stress due to the polyamide microfibers ingestion, the activity of the antioxidant enzyme catalase (CAT) was studied. To do so, the gills and the digestive glands of the mussels from the control tanks and from the polyamide different concentrations tanks were weighted, homogenized in an ice cold buffer and then centrifuged at 10,000 g for 10 min at 4ºC as described below in further detail.

2.8.1. Tissue Homogenates

The mantle, gills and digestive glands were homogenized with an ice-cold Tris-HCl buffer (50 mM) at pH 7.64 containing Saccharose (250 mM) and $MgCl_2$ hexahydrate anhydrous (5mM) (Bebianno *et al*., 2018) with a ratio of 1:5 (w:v; weight of the tissue: volume of the buffer). A mechanical grinder (BRAND?) and an ultrasonic homogenizer (Qsonica Q55 Sonicator, United States of America) were used to disrupt the cells membranes and release the cell contents into the solution. Afterwards, a centrifugation at 10000 g for 30 min at 4ºC (Thermo Scientific Heraeus Biofuge Stratos, Germany) was executed to precipitate debris and large cell

particles. The supernatant was collected in 1.5 mL microtubes and kept refrigerated throughout and then frozen at 80ºC.

2.8.2. Biomarker Analysis: Catalase (CAT) Activity

Catalase (CAT) is expressed in most of the organisms' tissues (Vives - Bauza, Starkov and Arumi, 2007) and is a fundamental enzyme in protecting the cells from oxidative damage by reactive oxygen species (ROS), as it catalyzes the degradation of H_2O_2, a harmful byproduct of metabolic processes into water and gaseous oxygen (Luck, 1954).

The CAT activity was measured using a method described by Greenwald (1985) in which the test principle is that the catalase enzyme can be measured by monitoring the consumption of H_2O_2 substrate at 240 nm (equation 2):

Equation (2) $2H_2O_2 \rightarrow 2H_2O + O_2$

The CAT assay is to be performed using a positive control in international units, 1 unit defined as "the amount of CAT necessary to decompose 1.0 µM of H_2O_2 per minute at pH 7.0 at 25ºC while concentrations of H_2O_2 fall from ~10.3 mM to 9.2 mM" (Weydert and Cullen, 2007). To do so, a phosphate buffer solution was prepared by mixing a dibasic solution of K_2HPO_4 (80 mM; M=174.18 g/mol) with a monobasic solution of KH2PO4 (M=136.09 g/mol) until a pH of 7.60 was achieved, mixing throughout with a magnetic stirrer and continuously monitoring the solution with a pH meter (VWR pHenomenal 1100 L, USA). The phosphate buffer was kept at room temperature before the analysis.

A solution of Hydrogen Peroxide was prepared by adding 15 µL of Hydrogen Peroxide. (35%) kept in the fridge to 10 mL of 80mM phosphate buffer pH 7.60 as described above. The volume of hydrogen peroxide was chosen to be 15 µL since it was previously tested with several volumes above, such as 170 µL, 85 µL and 25 µL. However, with these larger volumes, the amount of substrate given to the enzyme in the homogenates was deemed to be too high and leading to inhibition of the enzyme catalytic action. Thus, the mixture used allowed a steady decline of substrate at optical densities within 1.5 and 0.5 O.D. when measured kinetically at a wavelength of 240nm in a microplate spectrophotometer (ThermoScientific Multiskan GO Microplate, Finland)

The samples were defrosted from the -80ºC ultrafreezer and maintained cold in ice during the procedures until the reading of the activity enzyme.

A volume of 10 µL of each sample was placed in replicate wells in a UV-ready microplate, followed by 190 µL of phosphate buffer over a cold platform. The plate was brought to the microplate reader platform and just prior to reading, 100 µL of the hydrogen peroxide/phosphate solution was added to all wells using an electronic multichannel pipette. This last solution, containing the catalase substrate, was carefully added seconds before the reading of the microplate under 240 nm in a spectrophotometer at 25ºC. The kinetic reading went on for 5 min each replicate, a blank with 300 µL phosphate buffer was done.

The catalase activity was measured according to equation (3) and presented in mmol/min/mg tissue:

Equation (3) $$CAT = \frac{\left(\frac{\Delta Abs}{EC}\right) x \left(\frac{Vtotal}{Vsample}\right)}{Wtissue}$$

Where: CAT is the catalase activity (mmol/min/mg tissue), **ΔAbs** is the absorbance at 240 nm, **EC** is the extinction coefficient of H_2O_2 (40 M-1 cm-1), $\mathbf{V_{total}}$ is the well total volume, $\mathbf{V_{sample}}$ is the volume of the sample and $\mathbf{W_{tissue}}$ is the weight of the tissue.

2.9. Statistical Analysis

For all responses studied, an initial exploratory analysis was done using graphical representations.

Several ANOVA models were considered, depending on the nature of the response variable namely: (1) use of data for individual mussels from each tank or an average value for the tank and (2) the availability of data along 3 time periods. In total, 3 explanatory variables were considered:

- LEVEL - the main factor (PAMF concentration in the water with 4 levels, 0-control, 30, 100 and 200 fibers/L),
- REP - the random effect of the tank (3 replicate per LEVEL x TIME) and
- TIME – the date of sampling (0, 15, 30 and 45 days).

When LEVEL and TIME were part of the model, the interaction term LEVEL x TIME was included. REP was considered nested in LEVEL or LEVEL x TIME.

All response variables were tested for homoscedasticity with respect to LEVEL. When considered appropriate, the response variable was transformed to obtain homoscedasticity or normality. The software packages SPSS and SAS were used.

3. RESULTS

3.2. Water properties

The water properties in each tank were monitored every other day along the experiment. The following parameters were measured: Temperature (ºC), Salinity, pH, O_2 (mg/ L) (Table 5).

Table 5 Water parameters in the tanks, mean, standard deviation, minimum and maximum values for individual tank measurements taken every other day, for each treatment level.

Variables	Level	Mean	StdDev	Min	Max
Temperature	0	22.96	2.88	16.6	30.3
(∘C)	30	22.76	2.82	16.4	29.5
	100	22.07	2.71	16.3	28.5
	200	22.01	2.87	16.2	28.2
Salinity	0	38.22	1.00	36.2	40.7
(psu)	30	38.30	1.02	36.3	41.2
	100	38.30	0.90	36.3	40.9
	200	38.34	0.97	36.3	41.0
pH	0	8.28	0.58	6.3	9.5
	30	8.27	0.54	6.3	9.0
	100	8.27	0.55	6.4	9.0
	200	8.26	0.56	6.3	9.0
O_2 (mg/L)	0	6.91	0.50	6.0	7.8
	30	7.01	0.47	6.0	7.9
	100	7.09	0.51	6.0	8.5
	200	7.04	0.46	6.0	8.0

The four variables measured, show consistency of among all treatment levels. Since the tanks were in open air, the fluctuations in temperature followed daily and temporal cycles with probable influence on oxygen levels. The fluctuation in salinity is the result of pumping water from the Ria Formosa that exhibits variations in salinity related with temperature, tidal cycle and freshwater input. The pH can also change as a result of incoming water properties and accumulation of the specimen's excretion.

3.3. Impact of PAMF on growth

The mean and standard deviation for each body measurement and treatment level are presented in Table 6 (shell size) and **Error! Reference source not found.** (weights).

Table 6 Mean and standard deviation of the body measurements (in mm) taken at the end of the experiment (day 45), n = 12 in all groups.

Treatment level	Length		Width		Height	
	Mean	StdDev	Mean	StdDev	Mean	StdDev
C	33.34	11.86	19.07	7.75	22.82	7.65
30	32.68	12.23	23.13	10.91	24.92	10.52
100	28.79	10.68	17.21	8.13	20.57	8.20
200	30.92	11.16	22.99	9.60	24.04	9.46

Table 7 Mean weight and standard deviation for the total, soft tissues and shell weight (in g) taken at the end of the experiment (day 45), n = 12 in all groups.

Treatment level	Total		Soft tissues (g)		Shell (g)	
	Mean	StdDev	Mean	StdDev	Mean	StdDev
C	11.0	4.11	1.31	2.57	6.01	2.29
30	11.0	3.86	0.67	0.40	6.23	2.59
100	7.5	2.81	0.33	0.10	3.25	1.75
200	5.8	1.91	0.26	0.19	2.31	0.72

The effect of PAMF concentration on growth was verified at day 45, with an ANOVA for the main factor LEVEL (concentration of fibers (0, 30, 100 and 200) and the secondary random factor REP (tank replicates) nested in LEVEL.

All response variables were previously tested for homogeneity of variance with a Levene´s test for the main factor. In case of rejection of the null hypothesis, a log-transformation was performed and the Levene´s test repeated. The p-values for the Levene´s as well as for the two components of the ANOVA model, LEVEL and REP(LEVEL), are presented in Table 8.

Table 8 Significance (p-values) of the statistical tests on growth. The Levene's statistic for homogeneity of variances was applied to the factor LEVEL (corresponding to PAMF concentrations). For the Nested ANOVA, the p-values of the two factors in the model are also shown. The tank replicates (REP) was assumed to be random and nested in the main factor LEVEL. Four individuals were observed in each tank and PAMF concentration (df level 3, df rep(level) 2x4 and df error 3x3x4).

Response	**Levene's test for main factor LEVEL**	**Nested ANOVA Model: LEVEL + REP(LEVEL)**	
		LEVEL	**REP(LEVEL)**
Length	0.7766	0.6825	0.0146
Width	0.1604	0.2641	0.1397
Height	0.5948	0.6100	0.0812
Log (total weight)	0.7402	<0.0001	0.4031
Log (tissue weight)	0.2932	0.0004	0.0746
Log (shell weight)	0.6398	<0.0001	0.5043

The Levene´s test indicated agreement with the assumption of homogeneity of variance in all cases (p-values between 0.1604 and 0.7766).

The significance of the factors in the ANOVA model was not consistent for all responses considered. For the three variables associated with shell dimension, PAMF concentration was not significant in all cases. For length the replicate effect was significant (p-value = 0.0146), an indication there is a lot of variability inside the PAMF levels.

Contrary to the body measurements, the variables associated with the weight, show a reduction with the increase in concentration of PAMF (**Error! Reference source not found.**). There is a clear significant difference with respect to PAMF level (p-values from 0.0004 to <0.0001) (**Error! Reference source not found.**). These results indicate a reduction in growth that affects both the soft and hard parts of the mussels. The replicate was not significant for all weight variables.

Since there are no differences in the shell measurements with PAMF concentration, but clear differences in shell weight, it can be concluded that PAMF impairs growth by reducing the shell thickness.

3.4. Impact of PAMF on survival

The number of dead individuals was counted and the dead individuals removed whenever the tanks were monitored (every other day). The cumulative mortality results show that, overall, the maximum number of deaths occurring in a treatment level was 5. The cumulative number of dead individuals per treatment level is presented in **Error! Reference source not found.**. The control groups had a total of 2 dead individuals and in the tanks for treatment levels 30, 100 and 200, the total number of dead individuals was, respectively, 5, 0 and 2. Given the initial number in the tank (60 x 3 replicates for any given treatment level), the mortality varied between 0% and 2.8%, with no specific pattern relative to time or treatment level.

Considering the cumulative mortality for all the tanks, it is possible to observe the expected an increase of percentage of mortality in the levels long time, but with values are too low to be considered as significant (Figure 7).

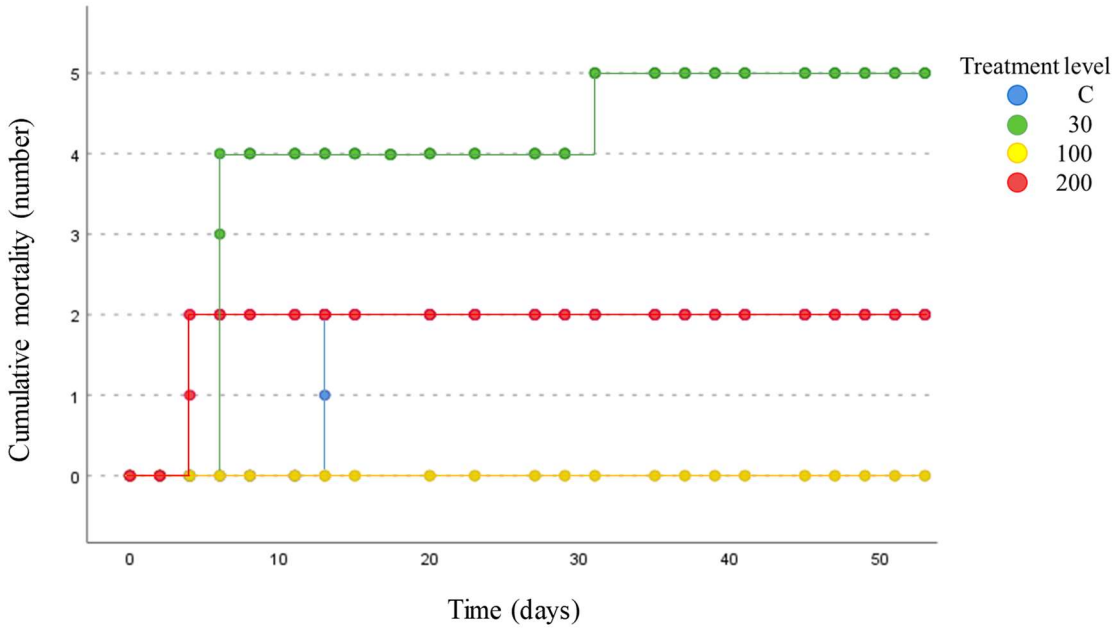


Figure 7 Cumulative mortality (in numbers) for each treatment level (initial number 180).

3.5. Accumulation of PAMF in the tissues

The number of fibers for each structure (A-mantle, B-gills, C-digestive grands, D-oral cavity and E-byssus), treatment level and point in time are presented in Figure 8. The mussel anatomy, identifying the structures where the fibers were counted, is shown in Figure 9.

The number of fibers in the different structure was similar (maximum values around 60), including the byssus where values were high in a single individual. There is no clear pattern of retention of the fibers (increase with time or level of PAMF), with the exception of the digestive glands where for the levels 30, 10 and 200 the retention of fibers increases from day 15 to 30 and then decreased to day 45.

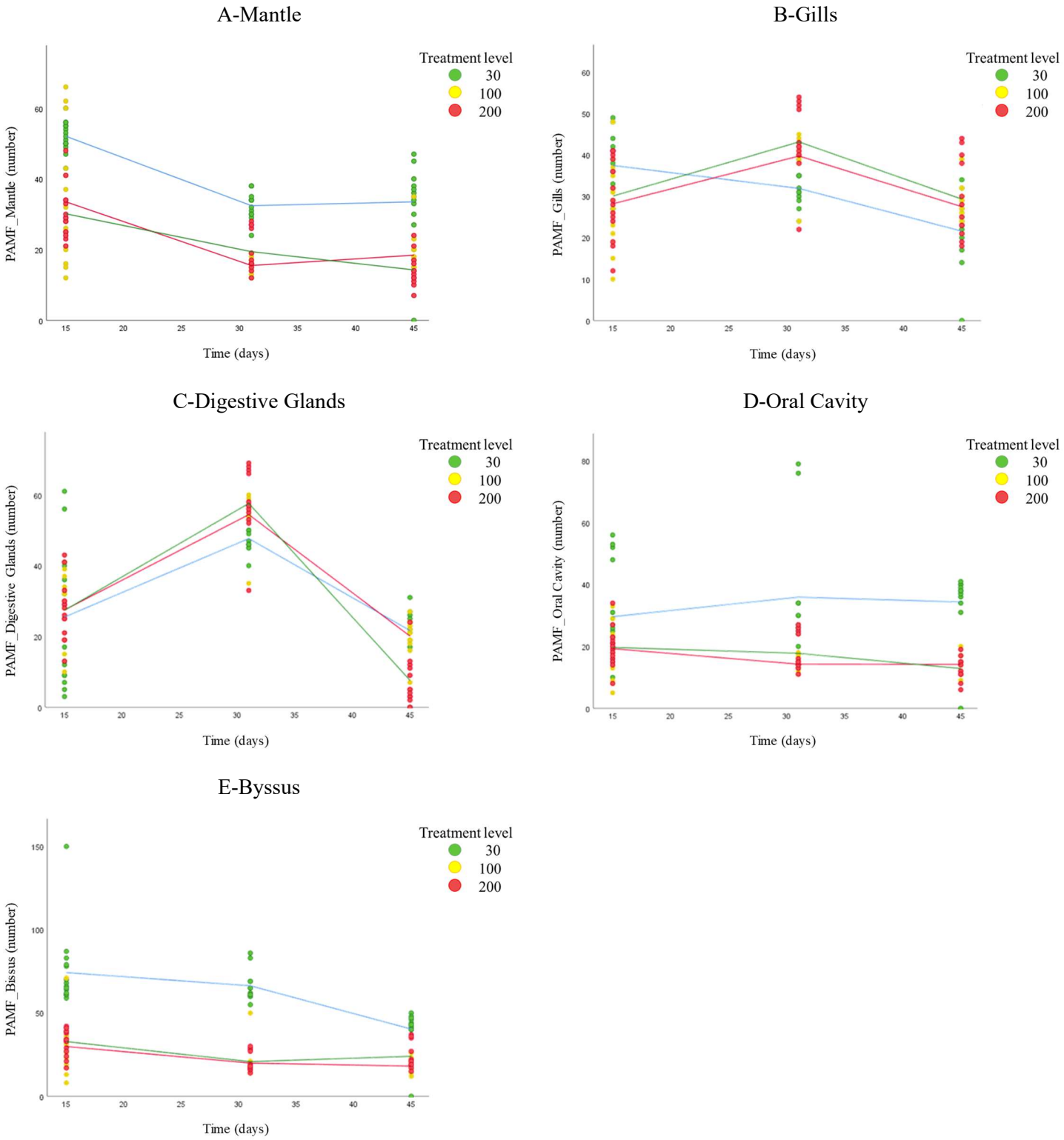


Figure 8 *N*umber of PAMF in the different tissues. For day 15, 15 individuals were dissected per level (5 per tank) and for days 30 and 45, 12 individuals were dissected per level (4 from each replicate).

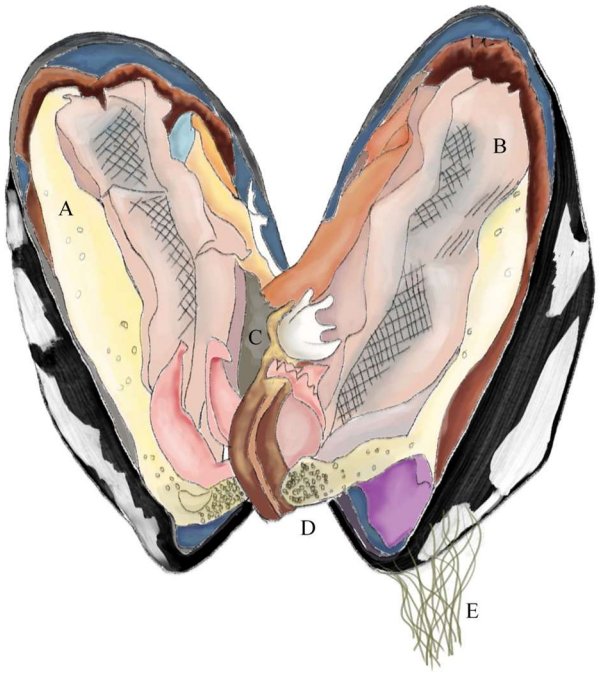


Figure 9 *Mussel anatomy, identifying the structures where the fibers were counted: A-mantle, B-gills, C-digestive grands, D-oral cavity and E-byssus. Design tools from KRITA – https://krita.org/en/.*

An ANOVA with two factors, LEVEL and TIME, was used to investigate the effect of PAMF concentration over time. The response variable (number of fibers in a given tissue type) was transformed by applying a square root transformation, used in counts (Fowler, Cohen and Jarvis, 1998). The control group was not included in the analysis, because it had only zero values. The results are resented Table 9.

Table 9 Statistical significance (p-values) of the ANOVA model: sqrt(number of fibers) = LEVEL + TIME for different tissue types (mantle, gills, digestive glands, oral cavity and byssus). Time refers to days 15, 30 and 45 of the experiment and LEVEL is the concentration of PAMF in the water 30, 100 and 200 PAMF/L. The degrees of freedom of the error terms are 112. The level control was not included in the analysis because it had only zero values.

Source of variation	**df**	**Mantle**	**Gills**	**Digestive glands**	**Oral cavity**	**Byssus**
LEVEL	2	<.0001	0.3326	0.1411	<.0001	<.0001
TIME	2	<.0001	<.0001	<.0001	0.3006	<.0001

The results confirm the concentration of fibers in the different tissues significantly change over time with the exception of the oral cavity. The analysis of Figure 10shows that fibers are more abundant at day 15 for the mantle and the byssus. For the oral cavity, the presence of fibers was similar along the three time periods (p = 0.3006 in **Error! Reference source not found.**).

The factor treatment level was significant for the tissues mantle, oral cavity and byssus. The analyses of the plots in Figure 10 show that in all three cases level 30 showed higher counts of fibers that levels 100 and 200, consistently over the three days of observation.

3.6. Impact of PAMF on food consumption

The total weight of fecal pellets in the tanks, considered an indicator of the amount of materials that passed through the gut, was counted ever time the water was changed, in each tank, at 22 points along the experiment (intervals of 2 days, except in weekends when the interval was 3 to 4 days). At the same time, the water parameters temperature, salinity, pH and O_2 were measured.

The weight of fecal pellets varied within the same range in all the treatment levels with a major pick occurring in all groups (**Error! Reference source not found.**) at day 30. For the rest of the time there was no pattern consistent with decreased digestibility due to the presence of fibers.

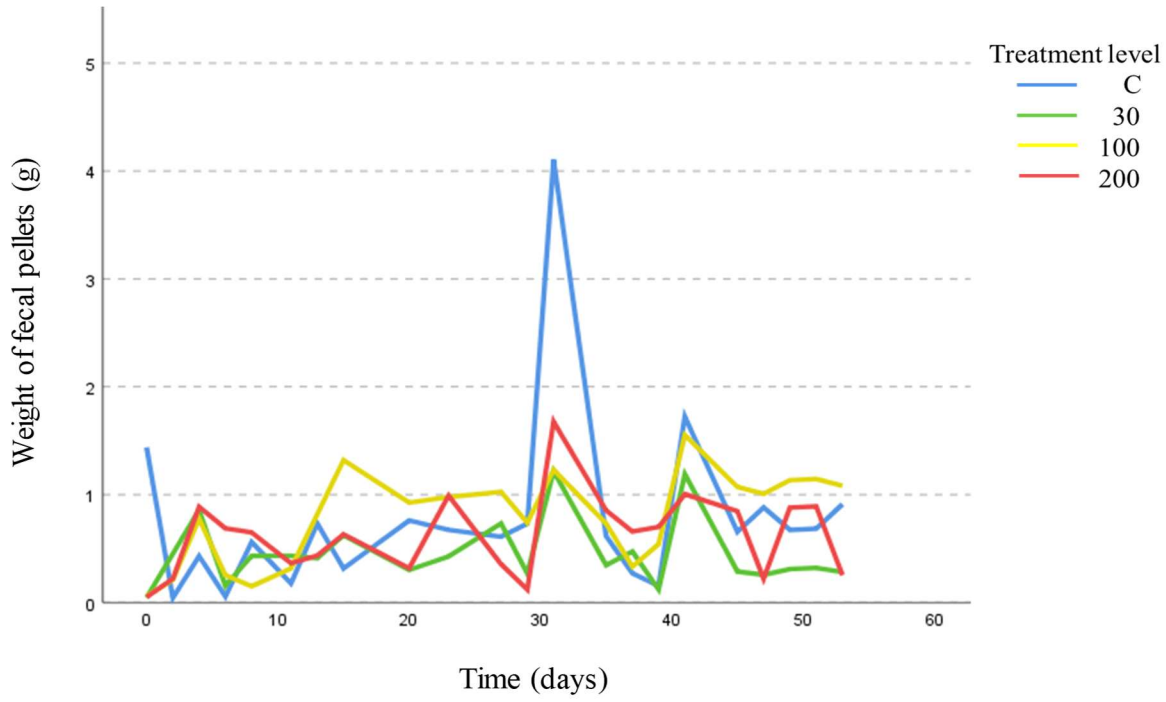


Figure 10 Total weight of fecal pellets present in the tanks along time (in g dry weight).

The peaks were probably related to external factors that were not considered/recorded. In general, there is no coincidence of such peaks of fecal pellet production with water parameters such as temperature, oxygen, salinity and pH levels (Figure 11).

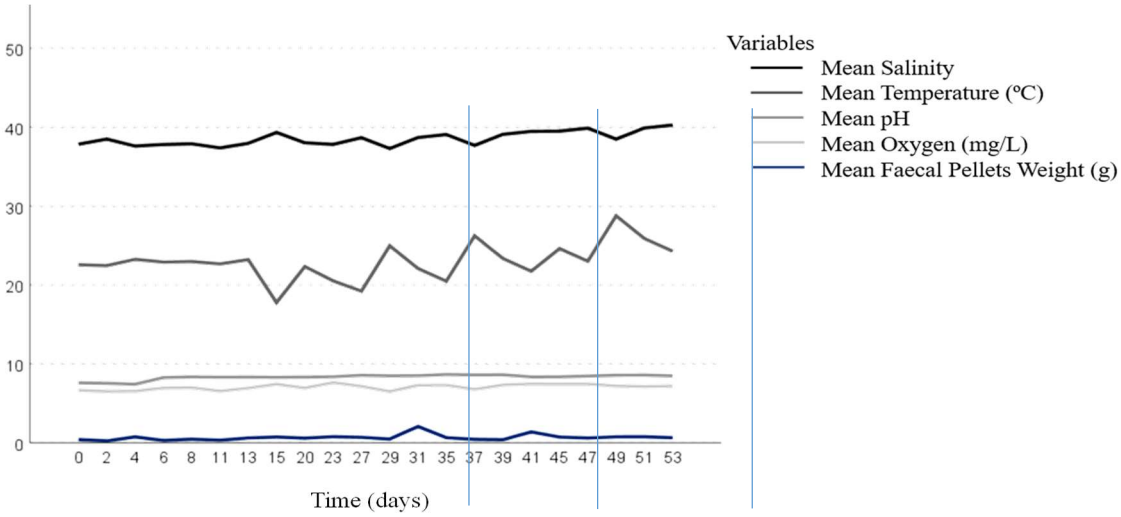


Figure 11 Mean values of the fecal pellets weight (in g), oxygen (in mg/L), pH, temperature (in ºC) and salinity along the time (in days).

A repeated measures linear model of log(weight of fecal pellet) as a function of PAMF concentration (factor LEVEL) and TIME (22 measurements over time) was used to investigate evolution of fecal pellet production along time (*Table 10*).

Table 10 Repeated measures ANOVA for the log(weight of fecal pellet) as a function of PAMF concentration (factor LEVEL) and TIME (22 measurements over time). The number of observations at each point in time was 12 (4 levels x 3 tank replicates).

Source of variation	DF	SS	MS	F Value	p-value	Exact p-values
LEVEL	3	5.04809	1.6827	22.48	0.0003	2.98E-04
Error(LEVEL)	8	0.59879	0.07485			
TIME	21	22.2984	1.06183	6.67	<.0001	1.46E-13
TIME x LEVEL	63	23.4293	0.37189	2.34	<.0001	8.16E-06
Error(TIME)	168	26.7479	0.15921			

There were significant differences among the fiber concentration (LEVEL) ($p = 0.0003$) and the TIME ($p < 0.0001$) (**Error! Reference source not found.**). The differences in fecal pellets weight are not consistent with the concentrations of PAMF since average higher values are found in the control (0.78g) and concentration 100 (79g) while the concentrations 30 and 200 had lower average values (0.45 and 0.61 respectively). The weight of fecal pellets produced is possibly related to an external factor that was not testes in this research study.

The significant difference related with TIME is due to the peaks at particular points in days, such as days 30 and 41, observed for all levels of PAMF. The interaction between LEVEL and TIME ($p = 0.0076$) is also significant because, within each point in time, the levels with higher and lower quantities of fecal pellets is not the same.

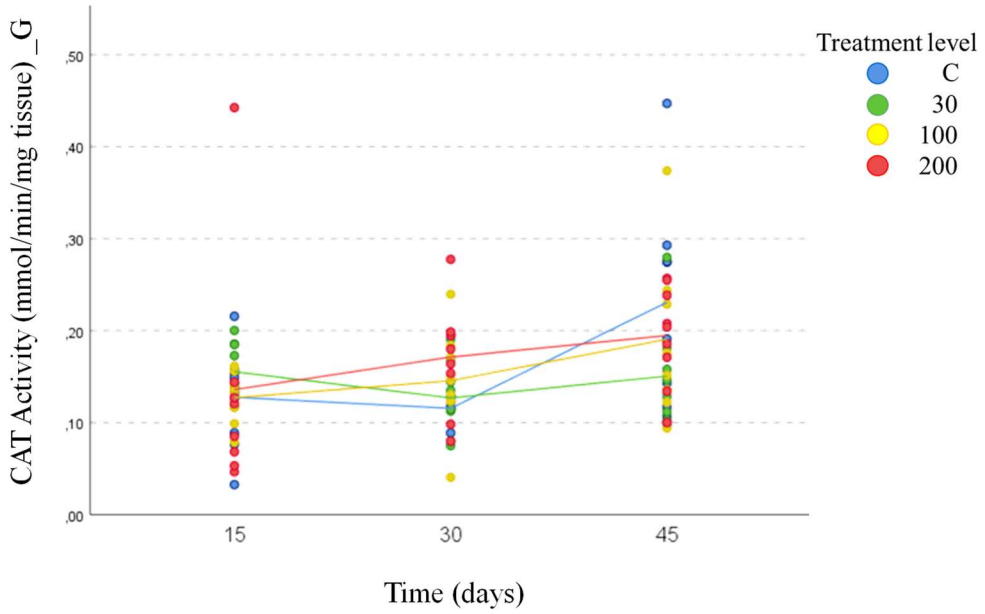
Due to a much smaller p-value for TIME (exact p-value = $1.46E^{-13}$) and interaction of TIME with LEVEL (exact p-value = $8.16E^{-16}$) (**Error! Reference source not found.**), time is the most important source of variation in fecal pellets weight.

3.7. Impact of PAMF on catalase activity

Catalase activity was measures at days 15, 30 and 45. The specimens, 3 per tank, were dissected and the digestive glands and gills were homogenized (separately foe each individual). Some of the specimens were lost and the number of replicates per LEVEL and TIME varied from 5 to 9. Graphs with CAT activity for each structure (digestive glands and gills), treatment level and time are presented in Figure 12 (A-digestive glands, B-gills).

The CAT activity in the digestive glands did change with the number of PAMF in the /*water, but maintained a similar range of values along the experiment. In the gills, there is a decrease of the CAT activity at the beginning of the experiment (day 15) and an increase after the day 30 with higher values at the end of the experiment (day 45) (Figure 12).

A-Gills



B-digestive grands

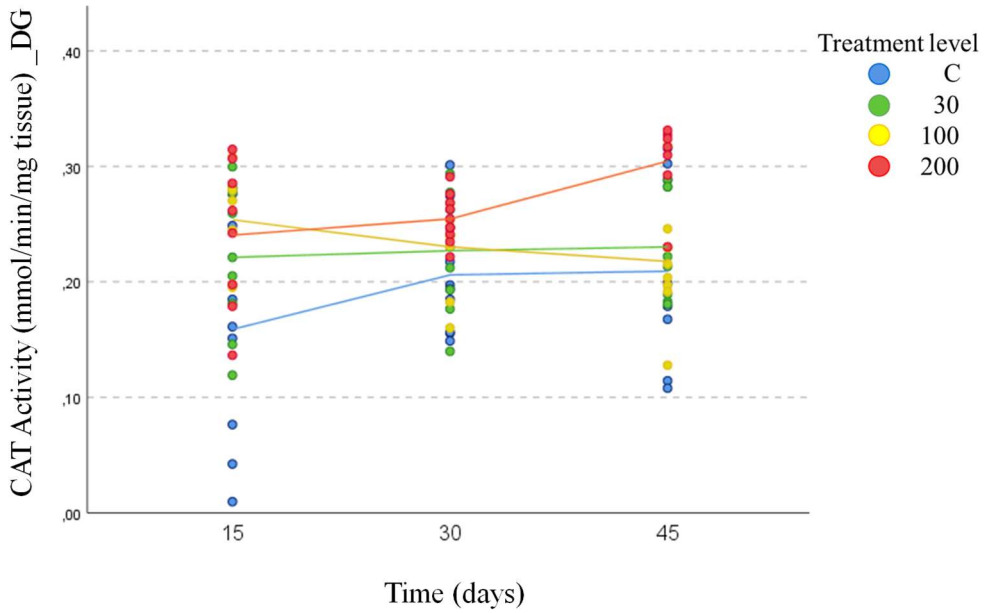


Figure 12 Catalase activity (mmol/min/mg tissue) measures at days 15, 30 and 45 of the experiment (n=15 per level and day).

A general linear model was used in which CAT activity was the dependent variable. The explanatory factors considered were LEVEL, TIME, their interaction and the tank effect (REP) considered nested in the crossing of LEVEL and TIME. This choice of model had the objective

of including all possible sources of variation that could be estimated. The result of the GLM model for the catalase activity in gills and digestive glands in presented in Table 11.

Table 11 General linear model for the CAT activity. A- gills, B-digestive glands.

A - Gills

Source	DF	SS	MS	F Value	p-value
Full Model	35	0.2231	0.0064	1.39	0.1250
LEVEL	3	0.0054	0.0018	0.40	0.7567
TIME	2	0.0737	0.0368	8.04	0.0008
LEVEL x TIME	6	0.0329	0.0055	1.20	0.3197
REP(LEVEL x TIME)	24	0.1190	0.0050	1.08	0.3879
ERROR	64	0.2932	0.0046		
Corrected TOTAL	99	0.5163			

B - Digestive glands

Source	DF	SS	MS	F Value	p-value
Full Model	35	0.2043	0.0058	1.89	0.0130
LEVEL	3	0.0733	0.0244	7.91	0.0001
TIME	2	0.0066	0.0033	1.08	0.3468
LEVEL x TIME	6	0.0320	0.0053	1.73	0.1282
REP(LEVEL x TIME)	24	0.0984	0.0041	1.33	0.1828
ERROR	66	0.2038	0.0031		
Corrected TOTAL	101	0.4082			

The CAT activity in the gills did not show any significant differences for the factors considered. The full model was not significant (p-value = 0.1250). The effect of TIME, when considered in isolation, was significant (p-value = 0.0008) but, in face of a non-significant full model, this significance should not be considered important.

For the digestive glands, the full model was significant (p-value = 0.0130) and the significance can be attributed to LEVEL, the concentration of PAMF. The effect can be seen in Figure 12*B,* where the red line, corresponding to the highest concentration of PAMF in the water, is consistently above all others and the control is consistently bellow. The lines in the middle, corresponding to concentrations 30 and 100, do not show a clear distinction from each other.

4. CHAPTER 4| DISCUSSION

Effects of natural environmental influences in mussels have been reported in terms of biochemical, physiological and behavioural responses of individuals (Blackstok, 1984; Khessiba *et al*., 2005). However, even though a wide range of vertebrates and invertebrates are known to ingest microparticles from plastic derivatives, the biological effects are not well known (Browne, 2008).

In this studied we have analyzed the impact of waterborne polyamide microfibers (PAMF) at different concentrations on the growth and physiological parameters in the mussel *M. galloprovincialis.* The concentrations used were 0 (control), 30, 100 and 200 PAMF/L (number of fibers 0.2 mm thick and length between 150 µm and 1000 µm).

Overall, the different levels of microfibers in the water did not reflect in impaired growth nor a major impact on the physiological functions examined. Some of the processes studied are discussed. The results also show that all tissues analyzed accumulate PAMF, although this accumulation varies by tissue with PAMF concentration and exposure time. The level of exposure appears to have an effect over the oxidative stress mechanisms, as catalase activity digestive glands was increased in animals exposed to higher concentrations of PAMF. However this result was not replicated in the gills.

The uptake of microplastics' derivatives could relate to the feeding strategy of bivalves as they are filterfeeders. It is possible that the microparticles can pass through the membranes after its ingestion and are incorporated in tissues such as lining of the gut cavity, then passing to other organs, possibly causing adverse effects (Wright *et al*, 2013; Browne *et al*, 2008).

Do PAMF have an impact on the digestive ability of the *Mytilus galloprovincialis*?

Fecal pellets were analyzed as an indicator of food uptake and digestive function. The weight of the fecal pellets fluctuated with time. Overall, its numbers at the end of the experiment were higher than the ones at the beginning, suggesting that the presence of PAMF did not have significant impact over the *M. galloprovincialis* digestion not causing blockage of the gut as described in another study about the ingestion of microfibers of plastic derivatives (Watts *et al, 2015*). However, the high variability does not allow to exclude an interaction with other factors, such as temperature, as the peaks in the amount of fecal pellets follow increases in water temperature

In this study, the results of the fecal pellet production were not conclusive. The effect of time was related with few peaks occurring all levels of PAMF and the differences in PAMF concentration did not show a pattern that could be explained by the fiber concentration.

The lack of a specific effect of PAMF concentration of the fecal pellet production indicates that ingestion and digestion were likely not compromised by the presence of the fibers despite the accumulation of PAMF in the oral cavity and digestive gland.

Bivalves are able to select high-quality organic particles and to reject inorganic material through pseudofeces (Ward and Shumway, 2004), even though this selection is not 100% efficient (Urban and Kirchman, 1992; Bayne et al., 1993). The bivalves' selection in terms of physical and chemical criteria is sill not well studied (Ward and Shumway, 2004).

Several authors have described previously the ingestion of different types, shapes and sizes of microplastics ingested by mussels in laboratory settings (e.g. (Browne et al., 2008; Cole et al., 2013; Thompson et al., 2004; von Moos et al., 2012; Ward and Shumway, 2004)) but there is already abundant evidence that wild populations accumulate microplastics(Van Cauwenberghe et al., submitted) and the same is true for farmed mussels (Mathalon and Hill, 2014).

The consumption and accumulation of plastics' derivatives microspheres on *M. edulis* have been confirmed to happen during filter-feeding, which is confirmed by the present results . In addition, this also indicates that the genus *Mytilus* is capable of injesting microfibers of different plastics' derivatives (Avio et al., 2015; Brillant and MacDonald, 2002; Browne et al., 2008; Von Moos et al., 2012; Wegner et al., 2012; Woods *et al.,* 2018).

A study from Woods *et al.,* 2018 showed a deplection of the filtration rates at concentrations of 3 microplastic fibers mL^{-1}, similar to what Xu *et al*. (2016) have concluded. Another study indicated an average of 0.36 ± 0.07 particles g^{-1}/tissue at time of consumption (pre-depuration values) on *M. edulis* from the North Sea. As for the oysters *Crassostrea gigas* cultured in the Atlantic Ocean, an average of 0.47 ± 0.16 particles g^{-1}/tissue are ingested (Cauwenberghe and Janssen, 2014).

We have not attempted to evaluate the effects of microplastic presence and concentration on filtration rates and whether PAMF would directly compete with the filtering and ingestion of microalgae and, to our surprise, the amount of PAMF in water is not directly related to tissue accumulation, namely in the filtering and digestive apparatus. It is possible that these organims may be able to selective eliminate the fibers, preventing excessive accumulation.

The filtration, rejection and/or ingestion of plastics' derivatives microfibers by mussels when in the natural environment can be influenced by consumer and prey-related factors not captured under laboratory experiments (Valiela, 1995).

Do PAMF affect its growth?

The growth of bivalves is related to multiple intrinsic (p.e. genetic), environmental (p.e. food) and social (p.e. competition with conspecifics) factors (Fuentes-Santos, Labarta and Fernández-Reiriz, 2018). Evaluation of population dynamics is dependent of the understanding of the nature and contribution of these factors to growth (Vincenzi *et al*., 2014). Several studies have been published regarding the effects of environmental conditions on bivalve growth (Gosling, 2003; Camacho, Labarta and Beiras, 1995).

In this study, *M. galloprovincialis* growth indicated by shell measurements such as length, width and height of the shell was not affected by PAMF concentration.We found, however, that

the relationship between these dimensions varied in all groups, which indicates that there is morphological plasticity of the shell material among the individuals. This plasticity has been well studied and documented from around the world, such as mussel *M. edulis* from Canada (Alunno-Bruscia *et al*, 2001; Lauzon-Guay *et al*, 2005), *M. galloprovincialis* from Spain (Cubillo *et al*, 2012), *Parreysia favidens* from India (Ramesha and Sophia, 2015) and *Hyriopsis (Limnoscapha) myersiana* from Thailand (Kovitvadhi *et al*, 2009) (Kovacic, 2017).

Despite that no changes were observed in exterior shell dimensions, there was a a decrease in weight of the soft tissues and the isolated shell with the increase of PAMF levels in the water. This indicates that although the presence of PAMF does not affect the size of the shell, it does change its weight, thus likely resulting in thinner shells. This was in fact the clearest effect, with statistical significance, from all the processes studied in this work. The process by which the presence of PAMF interacts with shell deposition is not clear and unfortunately it was not possible to characterize its mineral contents to evaluate if specific ions were depleted. A possible impairment of ion uptake cannot be ruled out, as this occurs via the digestive process and gills, but mostly in the mantle, the principal tissue involved in shell formation, creating the adequate medium for calcium carbonate deposition. All these tissues accumulated PAMF.

Impairment of adequate growth or even decrease of shell thickness was observed in a study by Balbi *et al* (2016), demonstrating affected shell layer growth during the early embryo development of the *M. galloprovincialis* in the presence of Bisphenol A (BPA) (a monomer used in plastic manufacturing). It actually showed that BPA can actively damage the shells, causing fragility; shell alterations, including rough surface, thinner valves, convex hinge, asymmetry and/or gap between valvae; and externalized velum (ciliated swimming and feeding organ), a typical feature of the pre-veliger stage (Balbi *et al*,2016).

Whether the presence of increasing different PAMF concentrations can affect the growth of the shell layers, affecting the robustness and compromising developmental processes along time remains to be seen. It is unlikely that the process is similar, since the actions of BPA occur mostly via its interaction with endocrine receptors involved in mineralization (Kang et al, 2007) and there is no evidence that PAMF can physically interact with these systems. However there is no information about potential PAMF-released chemicals or its effect in absorbing and concentrating water pollutants that may themselves interact with the organisms' physiology.

In parallel to this effect over shell thickness, there was also a reduction of the mass of soft tissue in organisms exposed to PAMF, which may indicate that metabolism may be elevated and thus less energy can be converted in body mass. Although we have made an initial attempt to determine oxygen consumption rates, technical and data collection difficulties precluded that the results can be used.

Other authors have found that changes in the environmental parameters may result in non-coincident rates of shell and soft tissue growth (Borrero and Hilbish, 1988). Therefore, changes in the soft tissues' weight are usually associated with seasonal variation in food availability (Bayne and Newel1 1983), with the reproductive cycle (Bayne and Worrall 1980) and with the energy storage and utilization patterns (Gabbott 1976, Barber and Blake 1981; Borrero and Hilbish, 1988). Opposite to that, the shells' growth only has partial dependence on metabolic carbon (Tanaka *et al* 1986) since it has lower organic content than the soft tissues (Josrgensen 1976, Price *et al* 1976) and its process depends on the deposition of the materials from the water (Wilbur and Saleuddin 1983; Borrero and Hilbish, 1988). Therefore, food availability and levels

of inorganic matter in the water have different effects in the soft tissues and shell tissues weight (Borrero and Hilbish, 1988).

This can indicate that the high PAMF levels can minimize the food availability to the mussels, so its soft tissues weight is affected. On the other hand, even though mussels do not depend only of the carbon generating metabolic rate for its shell development, the presence of PAMF can influence sufficiently the thickness of the shells in terms of metabolism or even in terms on inorganic carbon availability to its deposition in the shells surface along time. This may lead to a negative energy balance (Hilbish and Koehn, 1985) in the width concomitant loss of both soft and shell tissue (Koehn *et al,* 1980), on contrary to the shell metrics that, of course, can not be lost (Hilbish, 1986).

The shell thickness has a high ecological importance in the life course of the mussels. Since its main defense mechanism is having a strong calcareous shell (Gutierrez *et al*, 2003), as the main decisive factors in presence of predators are the size of mussel shells and thickness of the shells, making smaller and mussels with low thickness shells more available as prey for a particular predator (Hughes and Dunkin, 1984; Hamilton *et al*, 1999; Nagarajan *et al*, 2002a,b).

Different predators use different techniques to crash and get the soft tissues of the mussels (Garza, 2005). The success of these techniques depends on shell thickness and its resistance to crash (Nagarajan *et al*, 2006). Therefore, if the shell thickness decrease with the increase of PAMF levels, it makes them easier to crack and may makes the mussels more susceptible to predation in the natural environment as (Nagarajan *et al*, 2006) since specialiezed predators attack weak thin-shelled mussels first (Cayford and Goss-Custard, 1990).

Do PAMF cause oxidative damage along time?

Mytilus galloprovincialis has been shown to be a useful model organism to access the biological impact of the polyamide microfibers. This demonstrates that it can provide an indication of the environmental level of the polyamide microfibers along time (Regoli and Principato, 1995).

Several studies studies (Stien *et al*, 1997; Amiard-Triquet *et al*, 1998; Dellali *et al*, 2001; Khessiba *et al*, 2001; Da Ros *et al*, 2002; Roméo *et al*, 2003) have been done in order to validate the use of biomarkers in biomonitoring programs (Khessiba *et al*, 2005). In these studies, catalase was measured as a potential indicators of chemical-mediated oxidative stress (Stegeman *et al,* 1992) caused by the polyamide microfibers.

In presence of environmental contaminants/pollutants, oxidative stress may occur, resulting from one electron transfer reactions. This can produce oxygen derived free radicals or the so called reactive oxygen species (ROS). The ROS can actively react with the cellular components of the mussels such as the hemocytes (phagocytic circulating blood cells) which play an important role in the internal defence mechanism of mussels (Winston *et al*, 1996). Analogous to mammals, molluscan hemolymph contain hemocytes that contribute to the defence mechanisms releasing ROS during active phagocytosis (Winston *et al*, 1996).To combat the ROS in the system, enzymatic antioxidants start to act, including the catalase.

The antioxidant enzyme catalase is involved in the removal of the main precursor of hydroxyl radical in aquatic organisms: hydrogen peroxide (ROS type), acting as a defence mechanism toward exogenous sources (Regoli and Giuliani, 2014).

There were some differences in the catalase antioxidant enzyme activity along the experiment. In digestive glands CAT activity increased with the increasing in PAMF concentration and with time. For the gills, the CAT activity did not show significant differences with PAMF concentration or over TIME.

This can indicate that elimination of potential deleterious reactive oxygen species, including the superoxide anion radical and H_2O_2 was properly executed by the CAT under the presence of different concentrations of PAMF and that there is a tendency to an oxidative damage with the increase of PAMF due to the necessity of increased CAT activity in the tissues. The increase of the catalase activity is usually followed by an array of other enzymes involved in oxidative stress cascades, which were not tested in this study, but should deserve further attention in other to determine which substrates are being used in energy consumption in relation to PAMF exposure.

The differences in CAT activity for the digestive glands' tissues can be linked to the fact that bivalve digestive glands are the main tissues for the digestion and assimilation processes (Faucet *et al*., 2004), including the enzymatic digestion, possessing the same phase I and lI pathways of xenobiotic metabolism as in other invertebrates (Livingstone, 1994), so the PAMF could affect more directly this tissue.

Other studies in lugworm (*Arenicola marina*), in algae (*Chlorella* and *Scenedesmus*) indicated the existence of oxidative stress caused by micro and nanoplastics by measuring the ROS production, as well as the antioxidant capacity methods (Bhattacharya *et al*, 2010; Browne *et al*, 2013). Further studies confirmed oxidative stress by determining antioxidants concentrations in the presence of plastics derivatives (Chen *et al*, 2014; Chen *et al*, 2016; Chen *et al*, 2017).

Implications of other enzymes involved in the neutralization of ROS (specifically, of H_2O_2) can not be yet clarified (Paul-Pont *et al,* 2016).

As indicated above, in order to have a better perception of the impact of PAMF in terms of oxidative stress, further studies regarding other antioxidant enzyme assays should be performed, such as superoxidase dismutase (SOD), glutathione peroxidase (GPx), glutathione s-transferase (GST), glutathione (GSH), acetylcholinesterase (AChE) and lipid peroxide (LPO – 586) as well as protein determinations.

The gills are one of the respiratory surfaces of the mussels (Bierbaum and Shumway, 1988) and have filtration function which makes it an initial target for contaminants/pollutents in the marine and aquatic environment (Faucet *et al*., 2004). One of the hypothesis for this study regarding the effects of the PAMF in respiration or O_2 uptake was that the PAMF could adhere in the surface of the gills or cause damage, resulting in impaired ability to extract oxygen from the water. However, the presence of microfibers wasn't enough to make critical damages in the gills individual filaments, although histological studies could give a better picture of the impacts. This

means that there is no evidence of reduction in the respiration capability or O_2 due to the presence of PAMF in *M. galloprovincialis*.

There are a variety of responses that can be used by the mussels to regulate the oxygen gas exchange at the gills, including the increase of the pumping/ventilation rate and the increase in efficiency of oxygen extraction from the water (Bierbaum and Shumway, 1988). In fact, there are three different levels of oxygen consumption in *Mytilus edulis* (a species close to *M. galloprovincialis* in terms of physiology) that were described by Bayne *et al* (1973): standard, routine and active (Bierbaum and Shumway, 1988). Clogging of the gills may have impacts on all three of these rates, impairing mussels from effectively obtaining the required oxygen, specially under active conditions.

Do PAMF cause mortality?

In this study, the presence of PAMF did not cause increased mortality of the *M. galloprovincialis,* a result in agreement with a study by Kovacic, 2017, concluding that this species has a high capacity to acclimate and adapt within its morphometric and physiological parameters.

5. CHAPTER 5| CONCLUSION

The existence of polyamide microfibers in the *M. galloprovincialis* water tanks did not had a highly significant impact on the mussels health along the 45 days of experiment. Presence of PAMF was not correlated with mortality nor with major alterations in digestability but it appear to impact negatively shell formation and to certain extent growth of the soft tissues. Interestingly, the accumulation of PAMF in these organisms was not directly related to their abundance in water, which may indicate that mussels may be able to remove or somehow avoid them. In fact, the assumption that microplastics' derivatives are harmful to organisms is being highly controlled by the power of the media industry. Quoting Shumway, Ward and Mladinich (2018): "Very few studies clearly and reliably demonstrate any negative impacts of microplastics on bivalve molluscs".

Despite the "ever increasing number of scientific reports on the occurrence of of microplastics in the marine environment and associated impacts on marine life" (Cauwenberghe and Janssen, 2014), only (Cauwenberghe and Janssen, (2014) decribed the possible consequences of marine microplastics for humans in which it was reported that the human consumption of an average portion of mussels (250 g wet weight), there is the injestion of around 90 particles and an average portion of 6 oysters (100 g wet weight) contains around 50 particles.

Nevertheless, the toxicity from PAMF and other microplastics' derivatives to the ecosystems must not be neglected due to the fact that they are still a major pollutant nowadays and their mechanical impact, if not physiological, has been demonstrated in food uptake (Woods et al, 2018).

Even though the contamination due to PAMF did not have an important role in the health of the *M. galloprovincialis* during this research, future management actions, regulations and legislation implementation regarding PAMF pollution needs to be addressed due to their prevalence as major pollutant.

Production and/or cultivation of bivalves, such as mussels and oysters is mainly performed in coastal areas in which these organisms grow on suspended ropes from rafts and or on structures built above the seabed (Cauwenberghe and Janssen, 2014). So they are placed sessile into a specific location and are in constant contact pollutents coming from inland, including plastics' derivatives while feeding on natural microalgae presente in the seawater (Cauwenberghe and Janssen, 2014).

Several causes for such high levels of PAMF in the sea were identified by Gold *et al* (2013) but there is still insufficient understanding of the main sources of PAMF contamination/pollution and lack of enforcement of existing standards (Bergmann, Gutow and Klages, 2015; Chen, 2015). Hence, management actions inside commercial, touristic and fishing vessels, could reinforce the reduction or elimination of marine litter such as PAMF. The participation and cooperation of states in national and international initiatives are mandatory to make it happen and to make possible the creation of national litter programs, giving emphasis to pollutants/contaminants such as PAMF.

More integrative studies must be executed in the future for a better understanding of this issue and to create universal methodologies since there is a lack in scientific rigor and acceptable methods of animal husbandry (Shumway, Ward and Mladinich, 2018). Per instance, Claessens *et al.* (2013) concluded that the use of concentrated HNO_3 to extract nylon has an efficiency of 0%,

since this technique results into a total destruction of this type of this plastic' derivative by the HNO_3 (Cauwenberghe and Janssen, 2014). This may be the reason why some published papers in this topic have questionable data derived from non-universal methods in absence of reliable assessment of the reported findings (Shumway, Ward and Mladinich, 2018).

6. CHAPTER 6| FUTURE PERSPECTIVES

Different adaptations, tests and experiments have been left for the future due to lack of time. These type of experiments are usually high time consuming, requiring an everyday verification of the quality conditions of the tanks for the animal models. Future work concerns deeper analysis of particular mechanisms, new proposals to try different methods, or simply curiosity.

In a close future, studies involving two sequential trials will be: Trial I and Trial II (Annex B).

Trial I consists in analyzing the remaining samples from this MSc thesis research study in terms of a more complete investigation regarding the growth and the oxidative stress. The ammonium (NH_3) excretion will be measured by accessing the ammonium concentration in 1.5 mL water sample of each tank (a spectrophotometry colorimetric method will be used) at two different stipulated hours with a spacing of 9 hours between. This samples were already collected and stored at – 4ºC. Then, to measure it.

The ammonium excretion rate (µmol NH_4^+/organism/hour) will be calculated according to the equation (5):

Equation (5) $[NH3f-NH3i]xVwc_T$

Where **[NH3f – NH3i]** is the difference between the ammonia concentrations of the final and initial samples; **Vwc** is the volume of water container (L) and **T** is the excretion time (h) (Urbina *et al,* 2014).

The energy from the ammonia excretion rate (µmol NH4+/day) will be converted to energy using the conversion factor of 0.000447kJ/µmol.

In addition, to further evaluate energy demands, proper respirometry techniques can be used to evaluate oxygen consumption in organism control or exposed to PAMF.

Additionally, the assays for the antioxidant superoxidase dismutase (SOD), glutathione peroxidase (GPx), glutathione s-transferase (GST), glutathione (GSH), acetylcholinesterase (AChE) and lipid peroxide (LPO – 586) enzymes will be performed together with the protein determinations. The main goal of these enzymatic activity performances will be to show how the antioxidant cycles are affected in the different types of tissues homogenate (digestive glands, gills and mantle) that were already prepared are stored at -80ºC.

The mineral content of the shells can be determined. Shells will be reduced to ashes and dissolved in H_2NO_3 and cations determined, to evaluate whether the seemingly thin shells in PAMF organism depend on inadequate mineralization.

The Trial II will be conducted to study if there are changes of the presence of PAMF when they are present inside a prey. So, a total of thirty six *Sparus aurata* individuals (up to 70g) will be individually marked with a floy-tag, placed in a total of twelve tanks (4 levels x 3 replicates) with 60-90 L with open circuit (9 fish each, tanks A through D followed by the number of replicate) and fed with control mussels for one week (Annex B). Upon that period, the fish will be fed with the remaining mussels from Trial I (1 mussel/fish per day) for one week as follows: tank A (0 fibers/L), tank B (30 fibers/L) tank C (100 fibers/L) and tank D (200 fibers/L) (Annex B). Feces will be collected and analyzed for the presence of PAMF. The growth of *S. aurata* will evaluated, the individuals sacrificed and taken to the laboratory to evaluate gastrointestinal tract content and oxidative stress (Annex B).

In a more distant future and after the previous future perspectives, it would be interesting to also conduct a similar trial to the Trial II, but instead of feeding the *Sparus aurata* with

contaminated mussels, feed it with contaminated aquaculture ration to see if there are any differences in how PAMF behave chemically with the presence of different organic matter.

Also, integrated study described previously could be undertaken on different organisms, since the *M. galloprovincialis* and *S. aurata* are just two of the organisms among the many available as model organisms.

Another future perspective that will complete the knowledge about the impact of PAMF, specifically on marine organisms, will be the better understanding of the persistency of PAMF in the digestive glands of long life marine organisms can change its chemical composition and how it can impact the health of the model animals.

REFERENCES

Altreuter, R. M. (2017). Microfibers, Macro problems: a resource guide and toolkit for understanding and tackling the problem of plastic microfiber pollution in our communities.

Alunno-Bruscia, M., Bourget, E. and Fréchette, M. (2001). Shell allometry and length-mass-density relationship for *Mytilus edulis* in an experimental food-regulated situation. *Marine Ecology Progress Series*, *219*, 177-188.

Amiard, J. C., Amiard-Triquet, C., Berthet, B. and Metayer, C. (1986). Contribution to the ecotoxicological study of cadmium, lead, copper and zinc in the mussel *Mytilus edulis*. *Marine Biology*, *90*(3), 425-431.

Amiard-Triquet, C., Altmann, S., Amiard, J.C., Ballan-Dufrançais, C., Baumard, P., Budzinski, H., Crouzet, C., Garrigues, P., His, E., Jeantet, A.Y. and Menasria, R. (1998). Fate and effects of micropollutants in the Gironde estuary, France: a multidisciplinary approach. In *Oceans, Rivers and Lakes: Energy and Substance Transfers at Interfaces* (pp. 259-279). Springer, Dordrecht.

Anderson, P. J., Warrack, S., Langen, V., Challis, J. K., Hanson, M. L. and Rennie, M. D. (2017). Microplastic contamination in lake Winnipeg, Canada. *Environmental pollution*, *225*, 223-231.

Arthur, C., J. Baker and H. Bamford (eds). (2009). Proceedings of the International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris. Sept 9-11, 2008. *NOAA Technical Memorandum NOS-ORandR-30*.

Avio, C. G., Gorbi, S. and Regoli, F. (2015). Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: first observations in commercial species from Adriatic Sea. *Marine environmental research*, *111*, 18-26.

Avio, C. G., Stefania Gorbi, Massimo Milan, Maura Benedetti, Daniele Fattorini, Giuseppe d'Errico, Marianna Pauletto, Luca Bargelloni and Francesco Regoli. (2015). Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environmental Pollution*, *198*, 211-222.

Au, S. Y., Bruce, T. F., Bridges, W. C. and Klaine, S. J. (2015). Responses of *Hyalella azteca* to acute and chronic microplastic exposures. *Environmental toxicology and chemistry*, *34*(11), 2564-2572.

Auker, L. A. (2010). The effects of *Didemnum vexillum* overgrowth on *Mytilus edulis* biology and ecology. *Doctoral Dissertations*, *581*. https://scholars.unh.edu/dissertation/581

Bagaev, A., Mizyuk, A., Khatmullina, L., Isachenko, I. and Chubarenko, I. (2017). Anthropogenic fibres in the Baltic Sea water column: Field data, laboratory and numerical testing of their motion. *Science of The Total Environment*, *599*, 560-571.

Balbi, T., Franzellitti, S., Fabbri, R., Montagna, M., Fabbri, E. and Canesi, L. (2016). Impact of bisphenol A (BPA) on early embryo development in the marine mussel *Mytilus galloprovincialis*: effects on gene transcription. *Environmental pollution*, *218*, 996-1004.

Barker, J. C. (1976). Growth efficiencies and factors controlling size in some mytilid bivalves. Especially *Mytilus edulis* L.: review and interpretation.

Barnes, D. K., Galgani, F., Thompson, R. C. and Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *364*(1526), 1985-1998.

Batel, A., Linti, F., Scherer, M., Erdinger, L. and Braunbeck, T. (2016). Transfer of benzo [a] pyrene from microplastics to *Artemia nauplii* and further to zebrafish via a trophic food web experiment: CYP1A induction and visual tracking of persistent organic pollutants. *Environmental toxicology and chemistry*, *35*(7), 1656-1666.

Bayne, B. L. (1973). Physiological changes in *Mytilus edulis* L. induced by temperature and nutritive stress. *Journal of the Marine Biological Association of the United Kingdom*, *53*(1), 39-58.

Bayne, B. L. and Newell, R. C. (1983). Physiological energetics of marine molluscs. In *The mollusca* (pp. 407-515). Academic Press.

Bayne, B. L. and Worrall, C. M. (1980). Growth and production of mussels *Mytilus edulis* from two populations. *Mar. Ecol. Prog. Ser*, *3*, 317-328.

Bayne, B. L., Iglesias, J. I. P., Hawkins, A. J. S., Navarro, E., Heral, M. and Deslous-Paoli, J. M. (1993). Feeding behaviour of the mussel, *Mytilus edulis*: responses to variations in quantity and organic content of the seston. *Journal of the Marine Biological Association of the United Kingdom*, *73*(4), 813-829.

Bebianno, M. J., Cardoso, C., Gomes, T., Blasco, J., Santos, R. S. and Colaço, A. (2018). Metal interactions between the polychaete Branchipolynoe seepensis and the mussel *Bathymodiolus azoricus* from Mid-Atlantic-Ridge hydrothermal vent fields. *Marine environmental research*, *135*, 70-81.

Bergmann, M., Gutow, L. and Klages, M. (Eds.). (2015). *Marine anthropogenic litter*. Springer.

Bhattacharya, P., Lin, S., Turner, J. P. and Ke, P. C. (2010). Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. *The Journal of Physical Chemistry C*, *114*(39), 16556-16561.

Bierbaum, R. and Shumway, S. E. (1988). Filtration and oxygen consumption in mussels, *Mytilus edulis*, with and without pea crabs, *Pinnotheres maculatus*. *Estuaries*, *11*(4), 264-271.

Bisswanger, H. (2014). Enzyme assays. *Perspectives in Science*, *1*(1-6), 41-55.

Blackstock, J. (1984). Biochemical metabolic regulatory responses of marine invertebrates to natural environmental change and marine pollution. *Oceanogr. Mar. Biol. Ann. Rev*, *22*, 263-313.

Bocchetti, Raffaella, Daniele Fattorini, Barbara Pisanelli, Simona Macchia, Lisa Oliviero, Fabiano Pilato, David Pellegrini and Francesco Regoli. (2008). Contaminant accumulation and biomarker responses in caged mussels, Mytilus galloprovincialis, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. *Aquatic Toxicology*, *89*(4), 257-266.

Booth, A. M., Hansen, B. H., Frenzel, M., Johnsen, H. and Altin, D. (2016). Uptake and toxicity of methylmethacrylate-based nanoplastic particles in aquatic organisms. *Environmental toxicology and chemistry*, *35*(7), 1641-1649.

Borchers, P. and Hutchings, L. (1986). Starvation tolerance, development time and egg production of *Calanoides carinatus* in the Southern Benguela Current. *Journal of Plankton Research*, *8*(5), 855-874.

Borrero, F. J. and Hilbish, T. J. (1988). Temporal variation in shell and soft tissue growth of the mussel *Geukensia demissa*. *Marine ecology progress series. Oldendorf*, *42*(1), 9-15.

Brillant, M. and MacDonald, B. (2002). Postingestive selection in the sea scallop (*Placopectenmagellanicus*) on the basis of chemical properties of particles. *Marine Biology*, *141*(3), 457-465.

Browne, M. A., Crump, P., Niven, S. J., Teuten, E., Tonkin, A., Galloway, T. and Thompson, R. (2011). Accumulation of microplastic on shorelines woldwide: sources and sinks. *Environmental science and technology*, *45*(21), 9175-9179.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M. and Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environmental science and technology*, *42*(13), 5026-5031.

Browne, M. A., Galloway, T. and Thompson, R. (2007). Microplastic—an emerging contaminant of potential concern?. *Integrated Environmental Assessment and Management: An International Journal*, *3*(4), 559-561.

Browne, M. A., Niven, S. J., Galloway, T. S., Rowland, S. J. and Thompson, R. C. (2013). Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Current Biology*, *23*(23), 2388-2392.

Buchanan, J.B. (1971). Pollution by synthetic fibres. *Mar. Pollut. Bull. 2, 23.*

Caceres-Martinez, J., Robledo, J. A. and Figueras Huerta, A. (1994). Settlement and post-larvae behaviour of *Mytitus galtoprovincialis*: field and laboratory experiments.

Camacho, A. P., Labarta, U. and Beiras, R. (1995). Growth of mussels (*Mytilus edulis galloprovincialis*) on cultivation rafts: influence of seed source, cultivation site and phytoplankton availability. *Aquaculture*, *138*(1-4), 349-362.

Cantillo, A. Y. (1998). Comparison of results of mussel watch programs of the United States and France with worldwide mussel watch studies. *Marine Pollution Bulletin*, *36*(9), 712-717.

Carpenter, E. J. and Smith, K. L. (1972). Plastics on the Sargasso Sea surface. *Science*, *175*(4027), 1240-1241.

Claessens, M., Van Cauwenberghe, L., Vandegehuchte, M. B. and Janssen, C. R. (2013). New techniques for the detection of microplastics in sediments and field collected organisms. *Marine pollution bulletin*, *70*(1-2), 227-233.

Casado-Martinez, M. C., Smith, B. D., Luoma, S. N. and Rainbow, P. S. (2010). Bioaccumulation of arsenic from water and sediment by a deposit-feeding polychaete (*Arenicola marina*): a biodynamic modelling approach. *Aquatic Toxicology*, *98*(1), 34-43.

Cayford, J. T. and Goss-Custard, J. D. (1990). Seasonal changes in the size selection of mussels, *Mytilus edulis*, by oystercatchers, *Haematopus ostralegus*: an optimality approach. *Animal Behaviour*, *40*(4), 609-624.

Cedervall, T., Hansson, L. A., Lard, M., Frohm, B. and Linse, S. (2012). Food chain transport of nanoparticles affects behaviour and fat metabolism in fish. *PloS one*, *7*(2), e32254.

Cerkvenik-Flajs, V., Fonda, I. and Gombač, M. (2018). Analysis and occurrence of bisphenol A in mediterranean mussels (Mytilus galloprovincialis) sampled from the Slovenian Coastal Waters of the North Adriatic Sea. *Bulletin of environmental contamination and toxicology*, *101*(4), 439-445.

Cesa, F. S., Turra, A. and Baruque-Ramos, J. (2017). Synthetic fibers as microplastics in the marine environment: a review from textile perspective with a focus on domestic washings. *Science of the Total Environment*, *598*, 1116-1129.

Chen, Q., Yin, D., Hu, X., Wang, R. and Zhang, C. (2014). The effect of nC60 on tissue distribution of ibuprofen in *Cyprinus carpio*. *Science of the Total Environment*, *496*, 453-460.

Chen, Q., Hu, X., Wang, R., Yuan, J. and Yin, D. (2016). Fullerene inhibits benzo (a) pyrene Efflux from *Cyprinus carpio* hepatocytes by affecting cell membrane fluidity and P-glycoprotein expression. *Aquatic Toxicology*, *174*, 36-45.

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D. and Hollert, H. (2017). Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Science of the total environment*, *584*, 1022-1031.

Claessens, M., De Meester, S., Van Landuyt, L., De Clerck, K. and Janssen, C. R. (2011). Occurrence and distribution of microplastics in marine sediments along the Belgian coast. *Marine Pollution Bulletin*, *62*(10), 2199-2204.

Clark, James R., Matthew Cole, Penelope K. Lindeque, Elaine Fileman, Jeremy Blackford, Ceri Lewis, Timothy M. Lenton and Tamara S. Galloway. (2016). Marine microplastic debris: a targeted plan for understanding and quantifying interactions with marine life. *Frontiers in Ecology and the Environment*, *14*(6), 317-324.

Cole, M., Lindeque, P., Halsband, C. and Galloway, T. S. (2011). Microplastics as contaminants in the marine environment: a review. *Marine pollution bulletin*, *62*(12), 2588-2597.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J. and Galloway, T. S. (2013). Microplastic ingestion by zooplankton. *Environmental science and technology*, *47*(12), 6646-6655.

Cole, M., Lindeque, P., Fileman, E., Halsband, C. and Galloway, T. S. (2015). The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod *Calanus helgolandicus. Environmental science and technology*, *49*(2), 1130-1137.

Cooper, G. M., Hausman, R. E. and Hausman, R. E. (2000). *The cell: a molecular approach* (Vol. 10). Washington, DC: ASM press.

Costa, M. F., Do Sul, J. A. I., Silva-Cavalcanti, J. S., Araújo, M. C. B., Spengler, Â. and Tourinho, P. S. (2010). On the importance of size of plastic fragments and pellets on the strandline: a snapshot of a Brazilian beach. *Environmental Monitoring and Assessment*, *168*(1-4), 299-304.

Cubillo, A. M., Peteiro, L. G., Fernández-Reiriz, M. J. and Labarta, U. (2012). Density-dependent effects on morphological plasticity of *Mytilus galloprovincialis* in suspended culture. *Aquaculture*, *338*, 246-252.

Dagg, M. (1977). Some effects of patchy food environments on copepods 1. *Limnology and Oceanography*, *22*(1), 99-107.

Da Ros, L., Meneghetti, F. and Nasci, C. (2002). Field application of lysosomal destabilisation indices in the mussel *Mytilus galloprovincialis*: biomonitoring and transplantation in the Lagoon of Venice (north-east Italy). *Marine environmental research*, *54*(3-5), 817-822.

Dellali, M., Romeo, M. and Aissa, P. (2001). Suivi annuel de l'activité catalase chez des moules et des palourdes originaires de la lagune de Bizerte. *Oceanologica acta*, *24*(3), 263-271.

Deisseroth, A. L. B. E. R. T. and Dounce, A. L. (1970). Catalase: Physical and chemical properties, mechanism of catalysis and physiological role. *Physiological reviews*, *50*(3), 319-375.

Dekiff, J. H., Remy, D., Klasmeier, J. and Fries, E. (2014). Occurrence and spatial distribution of microplastics in sediments from Norderney. *Environmental Pollution*, *186*, 248-256.

Derraik, J. G. (2002). The pollution of the marine environment by plastic debris: a review. *Marine pollution bulletin*, *44*(9), 842-852.

Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K. A. and Corsi, I. (2014). Accumulation and embryotoxicity of polystyrene nanoparticles at early stage of development of sea urchin embryos *Paracentrotus lividus*. *Environmental science and technology*, *48*(20), 12302-12311.

De Vooys, C. G. N. (1987). Adaptation to anaerobic metabolism in two mussel species, *Mytilus edulis* and *Mytilus galloprovincialis*, from the tidal zone at Arcachon Bay, France. *Netherlands journal of sea research*, *21*(1), 17-23.

De Witte, B., Devriese, L., Bekaert, K., Hoffman, S., Vandermeersch, G., Cooreman, K. and Robbens, J. (2014). Quality assessment of the blue mussel (*Mytilus edulis*): comparison between commercial and wild types. *Marine pollution bulletin*, *85*(1), 146-155.

De Zwaan, A. and Wijsman, T. C. M. (1976). Anaerobic metabolism in bivalvia (Mollusca) characteristics of anaerobic metabolism. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *54*(3), 313-323.

De Zwart, L. L., Meerman, J. H., Commandeur, J. N. and Vermeulen, N. P. (1999). Biomarkers of free radical damage: applications in experimental animals and in humans. *Free Radical Biology and Medicine*, *26*(1-2), 202-226.

Donaghay, P. L. and Small, L. F. (1979). Food selection capabilities of the estuarine copepod *Acartia clausi*. *Marine Biology*, *52*(2), 137-146.

Dos Santos, J. and Jobling, M. (1991). Gastric emptying in cod, *Gadus morhua* L.: emptying and retention of indigestible solids. *Journal of Fish Biology*, *38*(2), 187-197.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological reviews*, *82*(1), 47-95.

Eerkes-Medrano, D., Thompson, R. C. and Aldridge, D. C. (2015). Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritisation of research needs. *Water research*, *75*, 63-82.

Elsevier (2018) *Science Direct*. *www.sciencedirect.com*. *Accessed on December 31st, 2018.*

Engler, R. E. (2012). The complex interaction between marine debris and toxic chemicals in the ocean. *Environmental science and technology*, *46*(22), 12302-12315.

Eriksen, Marcus, Sherri Mason, Stiv Wilson, Carolyn Box, Ann Zellers, William Edwards, Hannah Farley and Stephen Amato. (2013). Microplastic pollution in the surface waters of the Laurentian Great Lakes. *Marine pollution bulletin*, *77*(1-2), 177-182.

Eriksen, Marcus, Laurent CM Lebreton, Henry S. Carson, Martin Thiel, Charles J. Moore, Jose C. Borerro, Francois Galgani, Peter G. Ryan and Julia Reisser. (2014). Plastic

pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PloS one*, *9*(12), e111913.

FAO (2020). Species Fact Sheets: *Mytilus galloprovincialis*. Document prepared by Fisheries and Aquaculture Department (unpublished - http://www.fao.org/fishery/species/3529/en).

Farrell, P. and Nelson, K. (2013). Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). *Environmental Pollution*, *177*, 1-3.

Faucet, J., Maurice, M., Gagnaire, B., Renault, T. and Burgeot, T. (2004). Isolation and primary culture of gill and digestive gland cells from the common mussel *Mytilus edulis*. *Methods in cell science*, *25*(3-4), 177-184.

Fenech, M. and Ferguson, L. R. (2001). Vitamins/minerals and genomic stability in humans.

Foley, C. J., Feiner, Z. S., Malinich, T. D. and Höök, T. O. (2018). A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates. *Science of the Total Environment*, *631*, 550-559.

Fowler, J., Cohen, L. and Jarvis, P. (1998). Regression analysis. *Practical Statistics for Field Biology. 2nd ed. Wiley, Chichester*, 142-164.

Fuentes-Santos, I., Labarta, U. and Fernández-Reiriz, M. J. (2018). Characterizing individual variability in mussel (*Mytilus galloprovincialis*) growth and testing its physiological drivers using Functional Data Analysis. *PloS one*, *13*(10), e0205981.

Gago, J., Carretero, O., Filgueiras, A. V. and Viñas, L. (2018). Synthetic microfibers in the marine environment: A review on their occurrence in seawater and sediments. *Marine pollution bulletin*, *127*, 365-376.

Galloway, T. S. and Lewis, C. N. (2016). Marine microplastics spell big problems for future generations. *Proceedings of the National Academy of Sciences*, *113*(9), 2331-2333.

Gardner, J. P. A. (1992). *Mytilus galloprovincialis* (Lmk) (Bivalvia, Mollusca): the taxonomic status of the Mediterranean mussel. *Ophelia*, *35*(3), 219-243.

Garza, C. (2005). Prey productivity effects on the impact of predators of the mussel, *Mytilus californianus* (Conrad). *Journal of Experimental Marine Biology and Ecology*, *324*(1), 76-88.

GESAMP, 2015. Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection. Microplastics in the Ocean. (Retrieved from). http://www.gesamp.org/microplastics-in-the-ocean—a-global-assessment—wg-40-brochure.

Gold, M., Mika, K., Horowitz, C., Herzog, M. and Leitner, L. (2013). Stemming the tide of plastic marine litter: A global action agenda.

Google earth V 6.2.2.6613. (December 31st, 2018). Ria Formosa, Faro, Portugal. 37.008318, -7.994535, SIO, NOAA, U.S. Navy, NGA, GEBCO. TerraMetrics 2018, DigitalGlobe 2018. http://www.earth.google.com [June 26, 2018].

Gosline, J., Lillie, M., Carrington, E., Guerette, P., Ortlepp, C. and Savage, K. (2002). Elastic proteins: biological roles and mechanical properties. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *357*(1418), 121-132.

Gosling, E. (2003). Bivalve Molluscs: Biology. *Ecology and Culture. Blackwell, Oxford, England*.

Grafical Sotware KRITA (https://krita.org/en/)

Graham, E. R. and Thompson, J. T. (2009). Deposit-and suspension-feeding sea cucumbers (Echinodermata) ingest plastic fragments. *Journal of Experimental Marine Biology and Ecology*, *368*(1), 22-29.

Greenwald, R. A. (2018). *Handbook Methods For Oxygen Radical Research: 0*. CRC press.

Gruner, H. E. (1993). Lehrbuch der speziellen Zoologie. *Arthropoda (ohne Insecta)*.

Gutiérrez, J. L., Jones, C. G., Strayer, D. L. and Iribarne, O. O. (2003). Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos*, *101*(1), 79-90.

Gutow, L., Eckerlebe, A., Giménez, L. and Saborowski, R. (2015). Experimental evaluation of seaweeds as a vector for microplastics into marine food webs. *Environmental science and technology*, *50*(2), 915-923.

Halliwell, B. and Gutteridge, J. M. (2015). *Free radicals in biology and medicine*. Oxford University Press, USA.

Hamilton, D. J., Nudds, T. D. and Neate, J. (1999). Size-selective predation of blue mussels (*Mytilus edulis*) by common eiders (*Somateria mollissima*) under controlled field conditions. *The Auk*, *116*(2), 403-416.

Hilbish, T. J. and Koehn, R. K. (1985). The physiological basis of natural selection at the LAP locus. *Evolution*, *39*(6), 1302-1317.

Hilbish, T. J. (1986). Growth trajectories of shell and soft tissue in bivalves: seasonal variation in *Mytilus edulis* L. *Journal of experimental marine biology and ecology*, *96*(2), 103-113.

Hughes, R. N. and Dunkin, S. D. B. (1984). Effect of dietary history on selection of prey and foraging behaviour among patches of prey, by the dogwhelk, *Nucella lapillus* (L.). *Journal of Experimental Marine Biology and Ecology*, *79*(2), 159-172.

Jambeck, Jenna R., Roland Geyer, Chris Wilcox, Theodore R. Siegler, Miriam Perryman, Anthony Andrady, Ramani Narayan and Kara Lavender Law. (2015). Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768-771.

Jeong, Chang-Bum, Eun-Ji Won, Hye-Min Kang, Min-Chul Lee, Dae-Sik Hwang, Un-Ki Hwang, Bingsheng Zhou, Sami Souissi, Su-Jae Lee and Jae-Seong Lee. (2016). Microplastic size-dependent toxicity, oxidative stress induction and p-JNK and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). *Environmental science and technology*, *50*(16), 8849-8857.

Jones, H. D., Richards, O. G. and Southern, T. A. (1992). Gill dimensions, water pumping rate and body size in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology*, *155*(2), 213-237.

Kang, J. H., Aasi, D. and Katayama, Y. (2007). Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. *Critical reviews in toxicology*, *37*(7), 607-625.

Kashiwada, S. (2006). Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*). *Environmental health perspectives*, *114*(11), 1697.

Kaposi, K. L., Mos, B., Kelaher, B. P. and Dworjanyn, S. A. (2014). Ingestion of microplastic has limited impact on a marine larva. *Environmental science and technology*, *48*(3), 1638-1645.

Kappus, H. (1987). Oxidative stress in chemical toxicity. *Archives of toxicology*, *60*(1-3), 144-149.

Kaur, H. and Halliwell, B. (1994). Evidence for nitric oxide-mediated oxidative damage in chronic inflammation Nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS letters*, *350*(1), 9-12.

Kershaw, P. J. (2016). Marine plastic debris and microplastics–Global lessons and research to inspire action and guide policy change. *United Nations Environment Programme (UNEP), Nairobi* http://ec.europa.eu/environment/marine/goodenvironmental-status/descriptor-10/pdf/Marine_plastic_debris_and_microplastic_technical_report_advance_copy.pdf.

Khessiba, A., Hoarau, P., Gnassia-Barelli, M., Aissa, P. and Roméo, M. (2001). Biochemical response of the mussel *Mytilus galloprovincialis* from Bizerta (Tunisia) to chemical pollutant exposure. *Archives of environmental contamination and toxicology*, *40*(2), 222-229.

Khessiba, A., Roméo, M. and Aïssa, P. (2005). Effects of some environmental parameters on catalase activity measured in the mussel (*Mytilus galloprovincialis*) exposed to lindane. *Environmental Pollution*, *133*(2), 275-281.

Kolandhasamy, P., Su, L., Li, J., Qu, X., Jabeen, K. and Shi, H. (2018). Adherence of microplastics to soft tissue of mussels: a novel way to uptake microplastics beyond ingestion. *Science of The Total Environment*, *610*, 635-640.

Koehn, R. K., Newell, R. I. and Immermann, F. (1980). Maintenance of an aminopeptidase allele frequency cline by natural selection. *Proceedings of the National Academy of Sciences*, *77*(9), 5385-5389.

Kovačić, I., Pavičić-Hamer, D., Kanduč, T. and Hamer, B. (2017). Adaptation of cultured mussel *Mytilus galloprovincialis* Lamarck, 1819 from the northern Adriatic Sea to nearby aquaculture sites and translocation. *Acta Adriatica*, *58*(2).

Kovitvadhi, S., Kovitvadhi, U., Sawangwong, P., Trisaranuwatana, P. and Machado, J. (2009). Morphometric relationship of weight and size of cultured freshwater pearl mussel, *Hyriopsis (Limnoscapha) myersiana*, under laboratory conditions and earthen pond phases. *Aquaculture international*, *17*(1), 57-67.

Lauzon-Guay, J. S., Hamilton, D. J. and Barbeau, M. A. (2005). Effect of mussel density and size on the morphology of blue mussels (*Mytilus edulis*) grown in suspended culture in Prince Edward Island, Canada. *Aquaculture*, *249*(1-4), 265-274.

Lawrence, R. A. and Burk, R. F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. *Biochemical and biophysical research communications*, *71*(4), 952-958.

LeDoux, S. P., Driggers, W. J., Hollensworth, B. S. and Wilson, G. L. (1999). Repair of alkylation and oxidative damage in mitochondrial DNA. *Mutation research*, *434*(3), 149-159.

Lee, J., Hong, S., Song, Y.K., Hong, S.H., Jang, Y.C., Jang, M., Heo, N.W., Han, G.M., Lee, M.J., Kang, D. and Shim, W.J. (2013). Relationships among the abundances of plastic debris in different size classes on beaches in South Korea. *Marine pollution bulletin*, *77*(1-2), 349-354.

Le Pennec, G. and Le Pennec, M. (2001). Evaluation of the toxicity of chemical compounds using digestive acini of the bivalve mollusc *Pecten maximus* L. maintained alive in vitro. *Aquatic toxicology*, *53*(1), 1-7.

Li, J., Yang, D., Li, L., Jabeen, K. and Shi, H. (2015). Microplastics in commercial bivalves from China. *Environmental pollution*, *207*, 190-195.

Livingstone, D. R. (1994). Recent developments in marine invertebrate organic xenobiotic metabolism. *Toxicol. Ecotoxicol. News*, *1*(3), 88-95.

Livingstone, D. R. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine pollution bulletin*, *42*(8), 656-666.

Luck, H. (1954). Quantitative determination of catalase activity of biological material. *Enzymologia* 17, *1*(31-40).

Luís, L. G., Ferreira, P., Fonte, E., Oliveira, M. and Guilhermino, L. (2015). Does the presence of microplastics influence the acute toxicity of chromium (VI) to early juveniles of the common goby (*Pomatoschistus microps*)? A study with juveniles from two wild estuarine populations. *Aquatic Toxicology*, *164*, 163-174.

Lusher, A. L., Burke, A., O'Connor, I. and Officer, R. (2014). Microplastic pollution in the Northeast Atlantic Ocean: validated and opportunistic sampling. *Marine pollution bulletin*, *88*(1-2), 325-333.

Mathalon, A. and Hill, P. (2014). Microplastic fibers in the intertidal ecosystem surrounding Halifax Harbor, Nova Scotia. *Marine pollution bulletin*, *81*(1), 69-79.

McCord, J. M. and Fridovich, I. (1969). Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). *Journal of Biological chemistry*, *244*(22), 6049-6055.

McDonald, J. H. and Koehn, R. K. (1988). The mussels *Mytilus galloprovincialis* and *M. trossulus* on the Pacific coast of North America. *Marine Biology*, *99*(1), 111-118.

McDonald, J. H., Seed, R. and Koehn, R. K. (1991). Allozymes and morphometric characters of three species of *Mytilus* in the Northern and Southern Hemispheres. *Marine Biology*, *111*(3), 323-333.

Mente, E. (2003). Effect of ration level on individual food consumption, growth and protein synthesis in the shore crab *Carcinus maenas*. *Nutrition, physiology and metabolism of crustaceans. Science Publishers, Enfield, NH*, 53-67.

Messinger, L. (2016). How your clothes are poisoning our oceans and food supply. *The Guardian. https://www. theguardian. com/environment/2016/jun/20/microfibers-plastic-pollution-oceans-patagonia-synthetic-clothes-microbeads. Accessed on December 31st, 2018.*

Miller, R. Z., Watts, A. J., Winslow, B. O., Galloway, T. S. and Barrows, A. P. (2017). Mountains to the sea: river study of plastic and non-plastic microfiber pollution in the northeast USA. *Marine pollution bulletin*, *124*(1), 245-251.

Mikhailov, A. T., Torrado, M. A. R. I. O. and Mendez, J. O. S. E. F. I. N. A. (2002). Sexual differentiation of reproductive tissue in bivalve molluscs: identification of male associated polypeptide in the mantle of *Mytilus galloprovincialis* Lmk. *International Journal of Developmental Biology*, *39*(3), 545-548.

Moretti, A., Pedini Fernandez-Criado, M., Cittolin, G. and Guidastri, R. (1999). Manual on hatchery production of seabass and gilthead seabream , Vol.1. FAO, Rome, Italy. 194.

Morton, B. R. I. A. N. (1983). Feeding and digestion in Bivalvia. *The Mollusca. Physiology Part*, *2*, 65-147.

Mulcahy, M. F. (2000). Culture of molluscan cells. *Aquatic invertebrate cell culture. Springer-Praxis, Chichester, UK*, 165-181.

Murray, F. and Cowie, P. R. (2011). Plastic contamination in the decapod crustacean *Nephrops norvegicus* (Linnaeus, 1758). *Marine pollution bulletin*, *62*(6), 1207-1217.

Nasser, F. and Lynch, I. (2016). Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on *Daphnia magna*. *Journal of proteomics*, *137*, 45-51.

Nagarajan, R., Goss–Custard, J. D. and Lea, S. E. G. (2002). Oystercatchers use colour preference to achieve longer-term optimality. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *269*(1490), 523-528.

Nagarajan, R., Lea, S. E. and Goss-Custard, J. D. (2002). Reevaluation of patterns of mussel (*Mytilus edulis*) selection by European oystercatchers (*Haematopus ostralegus*). *Canadian Journal of Zoology*, *80*(5), 846-853.

Nagarajan, R., Lea, S. E. and Goss-Custard, J. D. (2006). Seasonal variations in mussel, *Mytilus edulis* L. shell thickness and strength and their ecological implications. *Journal of experimental marine biology and ecology*, *339*(2), 241-250.

Nel, H. A. and Froneman, P. W. (2015). A quantitative analysis of microplastic pollution along the south-eastern coastline of South Africa. *Marine pollution bulletin*, *101*(1), 274-279.

Ogonowski, M., Schür, C., Jarsén, Å. and Gorokhova, E. (2016). The effects of natural and anthropogenic microparticles on individual fitness in *Daphnia magna*. *PloS one*, *11*(5), e0155063.

Oliveira, M., Gravato, C. and Guilhermino, L. (2012). Acute toxic effects of pyrene on *Pomatoschistus microps* (*Teleostei, Gobiidae*): Mortality, biomarkers and swimming performance. *Ecological Indicators*, *19*, 206-214.

Oliveira, M., Ribeiro, A., Hylland, K. and Guilhermino, L. (2013). Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecological Indicators*, *34*, 641-647.

Ory, N. C., Sobral, P., Ferreira, J. L. and Thiel, M. (2017). Amberstripe scad *Decapterus muroads*i (Carangidae) fish ingest blue microplastics resembling their copepod prey along the coast of Rapa Nui (Easter Island) in the South Pacific subtropical gyre. *Science of the Total Environment*, *586*, 430-437.

Paglia, D. E. and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of laboratory and clinical medicine*, *70*(1), 158-169.

Paul-Pont, I., Lacroix, C., Fernández, C.G., Hégaret, H., Lambert, C., Le Goïc, N., Frère, L., Cassone, A.L., Sussarellu, R., Fabioux, C. and Guyomarch, J. (2016). Exposure of marine mussels *Mytilus spp*. to polystyrene microplastics: toxicity and influence on fluoranthene bioaccumulation. *Environmental Pollution*, *216*, 724-737.

Peng, G., Zhu, B., Yang, D., Su, L., Shi, H. and Li, D. (2017). Microplastics in sediments of the Changjiang Estuary, China. *Environmental Pollution*, *225*, 283-290.

Petersen, A. (2016). EFSA Panel on Contaminants in the Food Chain (CONTAM). Presence of microplastics and nanoplastics in food, with particular focus on seafood. Scientific

Opinion on the risks to animal and public health and the environment related to the presence of nickel in feed. *Efsa Journal*, *14*(6), e04501.

Pirc, U., Vidmar, M., Mozer, A. and Kržan, A. (2016). Emissions of microplastic fibers from microfiber fleece during domestic washing. *Environmental Science and Pollution Research*, *23*(21), 22206-22211.

Plastic Europe (2013). APME: Analysis of Plastic Production, Demand and Recovery in Europe. *Association of plastic manufacturers, Brussels (BE) (Retrieved from). http://www.plasticseurope.org/documents/document/20131014095824-final_plastics_the_facts_2013_published_october2013.pdf.*

Powell, M. D. and Berry, A. J. (1990). Ingestion and regurgitation of living and inert materials by the estuarine copepod *Eurytemora affinis* (Poppe) and the influence of salinity. *Estuarine, Coastal and Shelf Science*, *31*(6), 763-773.

Ramesha, M. M. and Sophia, S. O. L. A. I. (2015). Morphometry, length-weight relationships and condition index of Parreysia favidens (Benson, 1862) (Bivalvia: Unionidae) from river Seeta in the Western Ghats, India. *Indian Journal of Fisheries*, *62*(1), 18-24.

Regoli, F. and Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Marine Environmental Research*, *93*, 106-117.

Regoli, F. and Principato, G. (1995). Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology*, *31*(2), 143-164.

Regoli, F., Giuliani, M. E., Benedetti, M. and Arukwe, A. (2011). Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. *Aquatic toxicology*, *105*(3-4), 56-66.

Ribeiro, Francisca, Ana R. Garcia, Beatriz P. Pereira, Maria Fonseca, Nélia C. Mestre, Tainá G. Fonseca, Laura M. Ilharco and Maria João Bebianno. (2017). Microplastics effects in *Scrobicularia plana*. *Marine pollution bulletin*, *122*(1), 379-391.

Richardson, C. A. (1989). An analysis of the microgrowth bands in the shell of the common mussel *Mytilus edulis*. *Journal of the Marine Biological Association of the United Kingdom*, *69*(2), 477-491.

Richter, C., Park, J. W. and Ames, B. N. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proceedings of the National Academy of Sciences*, *85*(17), 6465-6467.

Rochman, C. M., Kurobe, T., Flores, I. and Teh, S. J. (2014). Early warning signs of endocrine disruption in adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from the marine environment. *Science of the Total Environment*, *493*, 656-661.

Roman, M. R. (1977). Feeding of the copepod *Acartia tonsa* on the diatom *Nitzschia closterium* and brown algae (*Fucus vesiculosus*) detritus. *Marine Biology*, *42*(2), 149-155.

Roméo, M., Hoarau, P., Garello, G., Gnassia-Barelli, M. and Girard, J. P. (2003). Mussel transplantation and biomarkers as useful tools for assessing water quality in the NW Mediterranean. *Environmental Pollution*, *122*(3), 369-378.

Rubbo, Homero, Rafael Radi, Madia Trujillo, Rossana Telleri, Balaraman Kalyanaraman, Stephen Barnes, Marion Kirk and Bruce A. Freeman. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *Journal of Biological Chemistry*, *269*(42), 26066-26075.

Sagert, J. and Waite, J. H. (2009). Hyperunstable matrix proteins in the byssus of *Mytilus galloprovincialis*. *Journal of Experimental Biology*, *212*(14), 2224-2236.

Salvador, C. F., Turra, A. and Baruque-Ramos, J. (2017). Corrigendum to" Synthetic fibers as microplastics in the marine environment: A review from textile perspective with a focus on domestic washings"[Sci. Total Environ. 598 (2017) 1116-1129]. *The Science of the total environment*, *603*, 836.

Saphoerster, J. (2008). The physiology of the blue mussel *(Mytilus edulis)* in relation to ocean acidification (Doctoral dissertation, Christian-Albrechts-Universität).

Sarà, G., Zenone, A. and Tomasello, A. (2009). Growth of *Mytilus galloprovincialis* (mollusca, bivalvia) close to fish farms: a case of integrated multi-trophic aquaculture within the Tyrrhenian Sea. *Hydrobiologia*, *636*(1), 129-136.

Sciences, H. and Zealand, N. (2002) 'Derraik. MPs in the marine env review. 2002', 44, pp. 842–852. doi: https://doi.org/10.1016/S0025-326X(02)00220-5.

Symbols for diagrams courtesy of the Integration and Application Network (ian.umces.edu/symbols).

Sivan, A. (2011). New perspectives in plastic biodegradation. *Current opinion in biotechnology*, *22*(3), 422-426.

Snell, T. W. and Hicks, D. G. (2011). Assessing toxicity of nanoparticles using *Brachionus manjavacas* (Rotifera). *Environmental toxicology*, *26*(2), 146-152.

Stadtman, E. R. and Levine, R. L. (2000). Protein oxidation. *Annals of the New York Academy of Sciences*, *899*(1), 191-208.

Stien, X., Percic, P., Gnassia-Barelli, M., Roméo, M. and Lafaurie, M. (1998). Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranean Sea. *Environmental Pollution*, *99*(3), 339-345.

Tanaka, N., Monaghan, M. C. and Rye, D. M. (1986). Contribution of metabolic carbon to mollusc and barnacle shell carbonate. *Nature*, *320*(6062), 520.

Taylor, M. L., Gwinnett, C., Robinson, L. F. and Woodall, L. C. (2016). Plastic microfibre ingestion by deep-sea organisms. *Scientific reports*, *6*, 33997.

Thompson, Richard C., Ylva Olsen, Richard P. Mitchell, Anthony Davis, Steven J. Rowland, Anthony WG John, Daniel McGonigle and Andrea E. Russell. (2004). Lost at sea: where is all the plastic?. *Science*, *304*(5672), 838-838.

Thompson, R. C., Moore, C. J., Vom Saal, F. S. and Swan, S. H. (2009). Plastics, the environment and human health: current consensus and future trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2153-2166.

Toulmond, A. and Tchernigovtzeff, C. (1984). Ventilation and respiratory gas exchanges of the lugworm *Arenicola marina* (L.) as functions of ambient PO_2 (20–700 torr). *Respiration physiology*, *57*(3), 349-363.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of physiology*, *552*(2), 335-344.

Urban Jr, E. R. and Kirchman, D. L. (1992). Effect of kaolinite clay on the feeding activity of the eastern oyster *Crassostrea virginica* (Gmelin). *Journal of experimental marine biology and ecology*, *160*(1), 47-60.

Urbina, M. A., Walsh, P. J., Hill, J. V. and Glover, C. N. (2014). Physiological and biochemical strategies for withstanding emersion in two galaxiid fishes. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, *176*, 49-58.

Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and environmental safety*, *64*(2), 178-189.

Valiela, I. and Valiela, I. (1995). Marine ecological processes. *Springer-Verlag, New York, NY, 696*.

Van Cauwenberghe, L. and Janssen, C. R. (2014). Microplastics in bivalves cultured for human consumption. *Environmental pollution*, *193*, 65-70.

Van Cauwenberghe, L., Claessens, M., Vandegehuchte, M. B., Mees, J. and Janssen, C. R. (2013). Assessment of marine debris on the Belgian Continental Shelf. *Marine pollution bulletin*, *73*(1), 161-169.

Van Cauwenberghe, L., Claessens, M., Vandegehuchte, M. B. and Janssen, C. R. (2015). Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (Arenicola marina) living in natural habitats. *Environmental Pollution*, *199*, 10-17.

Von Moos, N., Burkhardt-Holm, P. and Köhler, A. (2012). Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environmental science and technology*, *46*(20), 11327-11335.

Vincenzi, S., Mangel, M., Crivelli, A. J., Munch, S. and Skaug, H. J. (2014). Determining individual variation in growth and its implication for life-history and population processes using the Empirical Bayes method. *PLoS Computational Biology*, *10*(9), e1003828.

Vives-Bauza, C., Starkov, A. and Garcia-Arumi, E. (2007). Measurements of the antioxidant enzyme activities of superoxide dismutase, catalase and glutathione peroxidase. *Methods in cell biology*, *80*, 379-393.

Vlahogianni, T., Dassenakis, M., Scoullos, M. J. and Valavanidis, A. (2007). Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. *Marine Pollution Bulletin*, *54*(9), 1361-1371.

Wang, J., Tan, Z., Peng, J., Qiu, Q. and Li, M. (2016). The behaviors of microplastics in the marine environment. *Marine Environmental Research*, *113*, 7-17.

Ward, J. E. and Shumway, S. E. (2004). Separating the grain from the chaff: particle selection in suspension-and deposit-feeding bivalves. *Journal of Experimental Marine Biology and Ecology*, *300*(1-2), 83-130.

Ward, J. E., Zhao, S., Holohan, B. A., Mladinich, K. M., Griffin, T. W., Wozniak, J. and Shumway, S. E. (2019). Selective Ingestion and Egestion of Plastic Particles by the Blue Mussel (*Mytilus edulis*) and Eastern Oyster (*Crassostrea virginica*): Implications for Using Bivalves as Bioindicators of Microplastic Pollution. *Environmental science and technology*, *53*(15), 8776-8784.

Watts, A. J., Lewis, C., Goodhead, R. M., Beckett, S. J., Moger, J., Tyler, C. R. and Galloway, T. S. (2014). Uptake and retention of microplastics by the shore crab *Carcinus maenas*. *Environmental science and technology*, *48*(15), 8823-8830.

Watts, A. J., Urbina, M. A., Corr, S., Lewis, C. and Galloway, T. S. (2015). Ingestion of plastic microfibers by the crab *Carcinus maenas* and its effect on food consumption and energy balance. *Environmental science and technology*, *49*(24), 14597-14604.

Watts, A. J., Urbina, M. A., Goodhead, R., Moger, J., Lewis, C. and Galloway, T. S. (2016). Effect of microplastic on the gills of the shore crab *Carcinus maenas*. *Environmental science and technology*, *50*(10), 5364-5369.

Wegner, A., Besseling, E., Foekema, E. M., Kamermans, P. and Koelmans, A. A. (2012). Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.). *Environmental Toxicology and Chemistry*, *31*(11), 2490-2497.

Weydert, C. J. and Cullen, J. J. (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nature protocols*, *5*(1), 51.

Wheeler, C. R., Salzman, J. A., Elsayed, N. M., Omaye, S. T. and Korte Jr, D. W. (1990). Automated assays for superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activity. *Analytical biochemistry*, *184*(2), 193-199.

Widdows, J. and Bayne, B. L. (1971). Temperature acclimation of *Mytilus edulis* with reference to its energy budget. *Journal of the Marine Biological Association of the United Kingdom*, *51*(4), 827-843.

Widdows, J., Burns, K. A., Menon, N. R., Page, D. S. and Soria, S. (1990). Measurement of physiological energetics (scope for growth) and chemical contaminants in mussels (*Arca zebra*) transplanted along a contamination gradient in Bermuda. *Journal of Experimental Marine Biology and Ecology*, *138*(1-2), 99-117.

Widdows, J. and Donkin, P. (1992). Mussels and environmental contaminants: bioaccumulation and physiological aspects. *The mussel Mytilus: Ecology, physiology, genetics and culture*, *25*, 383-424.

Widdows, J. and Johnson, D. (1988). Physiological energetics of *Mytilus edulis*: scope for growth. *Marine Ecology Progress Series*, 113-121.

Widdows, J., Nasci, C. and Fossato, V. U. (1997). Effects of pollution on the scope for growth of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon, Italy. *Marine Environmental Research*, *43*(1-2), 69-79.

Widdows, J., Donkin, P., Evans, S. V., Page, D. S. and Salkeld, P. N. (1995). Sublethal biological effects and chemical contaminant monitoring of *Sullom Voe* (Shetland) using mussels (*Mytilus edulis*). *Proceedings of the Royal Society of Edinburgh, Section B: Biological Sciences*, *103*, 99-112.

Widdows, J., P. Donkin, M. D. Brinsley, S. V. Evans, P. N. Salkeld, A. Franklin, R. J. Law and M. J. Waldock. (1995). Scope for growth and contaminant levels in North Sea mussels *Mytilus edulis*. *Marine Ecology Progress Series*, *127*, 131-148.

Wilbur, K. M. and Saleuddin, A. S. M. (1983). Shell formation. In *The molusca, Academic Press.*235-287.

Winston, G. W., Moore, M. N., Kirchin, M. A. and Soverchia, C. (1996). Production of reactive oxygen species by hemocytes from the marine mussel, *Mytilus edulis*: lysosomal localization and effect of xenobiotics. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *113*(2), 221-229.

Woods, M. N., Stack, M. E., Fields, D. M., Shaw, S. D. and Matrai, P. A. (2018). Microplastic fiber uptake, ingestion and egestion rates in the blue mussel (*Mytilus edulis*). *Marine pollution bulletin*, *137*, 638-645.

WoRMS Editorial Board (2019). World Register of Marine Species. Available from http://www.marinespecies.org at VLIZ. Accessed 2019-11-08. doi:10.14284/170

Wright, S. L., Thompson, R. C. and Galloway, T. S. (2013). The physical impacts of microplastics on marine organisms: a review. *Environmental pollution*, *178*, 483-492.

Xu, X. Y., Lee, W. T., Chan, A. K. Y., Lo, H. S., Shin, P. K. S. and Cheung, S. G. (2017). Microplastic ingestion reduces energy intake in the clam Atactodea striata. *Marine pollution bulletin*, *124*(2), 798-802.

Yonge, C.M., 1947. The pallial organs in the aspidobranch gastropoda and their evolution throughout the mollusca. Phil. Trans. R. Soc. Ser. B, Vol. 232, pp. 443-518