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MSc Oceanography and Management of the Marine Environment

Effects of microplastics on mussels *Mytilus galloprovinciallis* from Greek waters

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Μεταπτυχιακό Ωκεανογραφίας και Διαχείρισης Θαλάσσιου Περιβάλλοντος

Η επίδραση των μικροπλαστικών στα μύδια *Mytilus galloprovinciallis* από ελληνικά νερά

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Abstract

Marine plastic pollution is increasing, causing microplastics (MPs) to be present in all marine environments, to travel great distances and accumulate into marine organisms. The aim of the study was to investigate the impact of MPs on *Mytilus galloprovincialis* in field conditions. Mussel samples were taken from Strofades, Gavdos, Zakynthos, Agios Georgios and Santorini considering these five places as clean from chemical pollutants. Mussels were analyzed for the detection of MPs and five different biomarkers were applied in order to evaluate any toxicological effects caused by MPs. The results did not confirm any significant responses of biomarkers that could be explained due to the presence of MPs, leading to the conclusion that other environmental factors are also responsible for changes in mussel's physiology.

Keywords: mussels, microplastics, biomarkers

Περίληψη

Η θαλάσσια ρύπανση εξαιτίας των πλαστικών αυξάνεται με αποτέλεσμα τα μικροπλαστικά (MPs) να βρίσκονται παντού στο θαλάσσιο περιβάλλον, να διανύουν μεγάλες αποστάσεις και τελικά να συσσωρεύονται σε θαλάσσιους οργανισμούς. Σκοπός της παρούσας μελέτης, ήταν η διερεύνηση της επίδρασης των μικροπλαστικών (MPs) στο είδος *Mytilus galloprovincialis*. Συλλέχθηκαν δείγματα μυδιών από τις περιοχές των Στροφάδων, της Γαύδου, της Ζακύνθου, του Άγιου Γεώργιου και της Σαντορίνης που βρίσκονται μακριά από πηγές ρύπανσης. Τα μύδια αναλύθηκαν για την ανίχνευση MPs και εφαρμόστηκαν πέντε διαφορετικοί βιοδείκτες προκειμένου να αξιολογηθούν τυχόν τοξικολογικές επιπτώσεις που προκαλούνται από MPs. Τα αποτελέσματα δεν επιβεβαίωσαν καμία σημαντική ανταπόκριση βιοδεικτών που θα μπορούσε να συσχετιστεί με τα MPs, οδηγώντας στο συμπέρασμα ότι άλλοι περιβαλλοντικοί παράγοντες είναι επίσης υπεύθυνοι για αλλαγές στη φυσιολογία του μυδιού.

Λέξεις κλειδιά: μύδια, μικροπλαστικά, βιοδείκτες

1.Introduction

Molluscs of the class Bivalvia are present in both marine and freshwater environments and the phylum Mollusca represents the second largest animal group (Plazzi & Passamonti, 2010). They are important for marine ecology, and they are often present in human diet (Orban *et al*., 2007). Bivalves contribute economically to the food industry as they get highly consumed, and they are also being used for their shells or the pearls they produce. Furthermore, mussels can be easily used as bioindicators for water quality as they tend to filter great amounts of water to get fed and they have the ability to accumulate contaminants (Farrington *et al*., 2016). They also have a wide geographical range and they can be tolerant to a wide range of temperature and salinity (Hudak, 2004). Bivalves are dominant filter-feeders on phytoplankton and other particles and their filtration rate can be influenced by environmental factors such as salinity, temperature, dissolved oxygen and concentration of organic and inorganic matter (Abel, 1976). These organisms live in areas where concentrations of inorganic and organic particles are extremely high, so they have evolved two different strategies in order to control the particles they receive. Some of them will reduce the clearance rate and they will only receive the amount of food they need and some others will increase the pseudofeces production so they will reject all the extra particles (Urban & Kirchman, 1992). As they filter, they increase water clarity and light penetration, contribute to nutrient cycling and overall, play important roles in ecosystem functioning (Castorani *et al*., 2015)

The Mediterranean mussel *Mytilus galloprovincialis* is endemic to the Mediterranean Sea and Black Sea and the Atlantic coast from Ireland to Marocco. This species is warm-water invasive, and it is believed that has displaced other native mussels becoming the dominant species in certain locations. It is dark blue or brown and usually, can be found in both rocky and sandy bottoms. It's the only mussel that can be found in open waters whereas, most mussels are stacked in bays and semi-closed areas. The maximum size of *Mytilus galloprovincialis* is 15cm, but usually we find it between 5 to 8 cm. A 5 cm long mussel can filter about 5 liters of water in one hour and when the environmental conditions are favorable for them, they grow to optimum size. An ideal condition for *M. galloprovincialis.* is when the sea water's temperature is about 15-25°C, the salinity 20-35‰ so they seem to be tolerant enough to face highly changing environmental conditions. They are found in forming beds at the bottom with density about 24,000 mussels/m². Their main diet consists of phytoplankton, but sometimes they can digest any organic matter. The species has 2 main reproductive cycles one in spring and one in autumn, but this is not certain as the spawning process can be affected easily from external factors like changes in water temperature and the availability of nutrients in their environment (Stankovic & Jovic, 2011). M. *galloprovincialis* has an important ecological role in the food web and a big socio-economic value due to its consumption as a seafood all around the world (Beyer *et al*., 2017). It is considered a suitable bioindicator of environmental contaminants and is widely used in marine pollution monitoring studies; especially the use of caged mussels reduces bias related

to the age and reproductive status of the organisms and allows better control of accumulation and biological effects of contaminants in a predetermined exposure period (Viarengo *et al*., 2007).

Plastic debris has been recognized as a severe pollution problem of the marine environment in the last decades and plastic particles have been found in oceans worldwide. Plastic is the most commonly used material in modern society as it supports all socio-economic activities and sectors (The Facts, 2020). It is proven that about 280 million tons of plastic are produced annually and plastic has become the main material of a lot of consumer products because of its light weight, the long-lasting duration, and the fact they are inexpensive. Approximately, 80% of plastics found in the water comes from the land and this makes urban coastal areas vulnerable to plastic pollution (Browne *et al*. 2011; Fisner *et al*. 2013). Aquaculture is also a main plastic source as the most used materials for fish production are plastic like the tanks, nets, ropes, pipes, buoys and cages (Wu *et al.*, 2020). Other sea-based sources of marine plastic litter include fishing and shipping. Plastic's main ecological disadvantage is that it doesn't eliminate in nature but only degrades into smaller pieces. It degrades very slowly, persisting in the environment for hundreds of years. Only 9 % of plastic waste is recycled, while 12 % is incinerated and 79 % ends up in landfills. This process usually takes place in coastal areas where the wave action is strong and the radiation is high (Barnes *et al.*, 2009). Many of those tons of plastic that end up in the oceans get trapped in circulating currents and they travel the world (Sigler, 2014). Approximately, 79% of plastics, finally will travel to the oceans and the ecological impact can be physical injuries and transfer of toxic chemicals to marine biota, or geographical transfer of microorganisms colonizing plastics surfaces (Allsopp *et al*. 2006; Cole *et al*. 2011). Plastic contamination has become hazardous to the entire marine ecosystem as a lot of organisms can easily ingest plastic or get trapped in it. The most abundant polymer found in marine environments is polyethylene (PE), but types like polystyrene (PS), polypropylene (PP), and polyvinyl chloride (PVC) can easily be found. Plastics are synthetic or semi-synthetic and they break into smaller pieces, or they are originally produced in tiny scale for commercial purposes, causing them to enter and accumulate in the tissues of many marine organisms. (LI *et al*., 2016). Microplastics (MPs) are plastic particles less than 5 mm in size and when they get absorbed by marine organisms can long term damage their organs and tissues (Arthur *et al*., 2009). The plastic items that usually produced at this size are production powders, cleaning products, industrial abrasives, etc. and they represent the primary MPs. Secondary MPs are produced from the fragmentation of plastic items into small ones (Andrady, 2017). They can be found in air, solid, water, food and humans' stools (Koelmans *et al.*, 2022). The direct or indirect ingestion of these particles by organisms in the ocean can happen via filtration and confusion with prey as a lot of marine animals like filter and deposit filters, detritivores and planktivores feed on organic matter in the size of MPs as well as via trophic transfer, (Wright *et al*., 2013).

MP ingestion has been found in a wide range of organisms including zooplankton, worms, shellfish, fish, seabirds and cetaceans (Fossi *et al*., 2018). Factors affecting the bioavailability of MPs into marine organisms is their size, density and colour. Their small size makes them easy for ingestion and accumulation in several tissues and their density is determining the type of the organisms that receives them as lighter MPs float and get absorbed by planktivores, whereas denser ones sink and get caught by deposit feeders. Filter-feeders are mostly prone to low-density MPs cause this type is more likely to float and get consumed by them (Albano *et. al*, 2021). The colour of the MPs can also be a trap for visual organisms when they resemble their prey (Wright *et al.*, 2013).
Depending on the shape of MPs, several main categories have been identified, each w ith a unique history. Different shapes include spheres, granular grains and agglomerat es, which are described as "primary MPs". Since these products are included in daily personal care products, they are very common as the use of these products is regular. Other shapes include fragments, fibers from washing machines and microfibers.

Bivalves are among the most studied organisms worldwide for assessing MP ingestion and especially mussels of the species *Mytilus galloprovincialis* are proposed as suitable bioindicators for monitoring MP ingestion and impacts on biodiversity in the Mediterranean Sea (Fossi *et al*. 2018, Li *et al*. 2019, Tsangaris *et al*., 2021).
Mussels as benthic filter-feeding organisms, are prone to ingesting MPs, mostly fibers and less fragments and pellets, due to their feeding technique, that can lead to physical harm and toxicity like inflammation, injure of their gills and digestive tract, oxidative stress, and decreased feeding rates (Balbi *et al.,* 2017; Zhang *et al*., 2019). MPs, due to their large surface area, also attract harmful hydrophobic pollutants like polychlorinated biphenyls (PCBs) and dichlorodirphenyltrichloroethane (DDT) (Zbyszewski and Corcoran 2011, EPA 2013). Furthermore, MPs tend to affect phytoplankton intake by the mussels with higher concentration leading to lower filtration levels (Woods *et al*., 2018). Previous studies have shown that nanoplatics or MPs <5μm can be accumulated in tissues and cells, whereas particles larger than 6μm are often being removed via pseudofeaces (Fernández and Albentosa, 2019; Gonçalves *et al*., 2019).

Ingestion, adherence and fusion are the 3 main stages that MPs follow when they enter the mussel's organism, but scientists still persist that there are numerous ways that, MPs can interact with shellfish (Kolandhasamy *et al*., 2018; Li *et al*., 2019b). MPs enter the organism through the inhalant siphons and then get trapped in the gills. The next part is either the MP to get assimilated over the gill epithelium or get transferred in the digestive system by endocytosis, so the main organs that contribute to the ingestion process are the gills, the intestine, the stomach and the siphons (Kolandhasamy *et al*., 2018). More recent studies have proven that MPs can also accumulate by adherence, for instance, on the surface of the mussel's foot (Kolandhasamy *et al*., 2018). After ingestion, MPs may remain in the gastrointestinal tract, be excreted through the stool, or transferred to body tissues by the epithelial cells of the gut (translocation). Translocation of MPs to the circulatory system (hemolymph) and potential redistribution to other tissues has been shown in mussels after short term laboratory exposure to polysterene MPs (Browne *et al*., 2008; GESAMP, 2016). Histological

changes, inflammatory response, reduction in byssus production or attachment strength, genotoxicity, reduced filtering activity and sometimes DNA damages are observed in mussels after exposure to 2,5 g/L polyethylene (Kolandhasamy *et al.,* 2018; Masiá *et al.,* 2021). Genotoxic effects include DNA strand breaks, nuclear anomalies and micronuclei due to the reactive oxygen species production and oxidative stress caused by MPs (Webb *et al.,* 2020; Zhang *et al*., 2019). The adverse effects of MPs on mussels have mostly been examined under laboratory conditions while relevant studies in field conditions are limited (Li *et al*., 2019, Balbi *et al*., 2017).

Biomarkers are commonly used tools for the evaluation of chemical contaminant effects and they can be also used to examine effects of microplastic ingestion. Biomarkers are functional measures of exposures to stressors that arise at cellular, biochemical, molecular and physiological level; they are changes measured mostly in cells or other parts like tissues and organs of an organism and are indicative of xenobiotic exposure and/or effect (Lam and Gray, 2003). It is generally believed that sub organism responses occur prior to alterations at the population and community levels so biomarkers can be predictive and anticipatory. Biomarkers enable integration of toxicant interactions in molecular or cellular targets resulting from exposure to complex mixtures of contaminants.

Biomarkers of oxidative stress and genotoxicity both respond to a wide range of contaminants as well as MPs. Oxidative stress can derive from increased production of reactive oxygen species (ROS) mediated by heavy metals and numerous organic contaminants that can be transported to marine organisms via microplastic ingestion. Furthermore, MPs consist of long hydrophobic chains which can absorb and accumulate several pollutants and cause the overproduction of ROS (Guo & Wang, 2019). ROS are reactive compounds that can cause cellular damage by reacting with biomolecules and generating several end-products such as malondialdehyde (MDA), carbonyl derivates and 8-hydroxy-2- deoxyguanosine (8-OHdG) (Sureda *et.al*, 2006). Among these are the superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) (Livingstone *et al*., 1992). The imbalance between production of ROS and antioxidant defenses may lead to oxidative stress manifested as oxidative damage of lipids, proteins, and DNA (Viarengo *et al*., 2007). Thus, changes in antioxidant defenses and oxidative damage are used as biomarkers of oxidative stress (Livingstone 2001). Genotoxic pollutants induce changes in the genetic material of marine biota, including DNA damage as well as gene and chromosomal alterations. Many types of environmental contaminants, such as metals, PAHs, polychlorinated biphenyls (PCBs), and pesticides, that can be adsorbed on MPs are known to exhibit genotoxic properties. Biomarkers indicating DNA and chromosomal damage are used to assess genotoxic effects in aquatic organisms (Ohe *et al*. 2004).

Catalase (CAT) is an enzyme of the antioxidant defense used as biomarker of oxidative stress (Livingstone, 2001). CAT is an extremely active catalyst for reduction of H_2O_2 to H_2O at high levels of H_2O_2, but at low levels it modulates the detoxification of other substances as phenols and alcohols through reactions coupled to the H_2O_2 reduction.

Glutathione S-tranferase (GST) is a Phase II detoxification enzyme involved in the conjugation and detoxification of organic compounds, and also plays a protective role against oxidative stress by catalyzing a selenium-dependent glutathione peroxidase. The enzyme response, for both CAT and GST to toxic chemicals, shows an initial increase due to the activation of enzyme synthesis followed by a decrease in enzymatic activity (Viarengo *et al*., 2007). On the other hand, lipid peroxidation (LPO) indicates the damage to cellular membrane lipids caused by ROS.

The micronucleus (MN) test, due to its simplicity, is one of the most applicable techniques to identify genomic alterations and has been shown to respond to MP exposure in aquatic organisms (Sun *et al*., 2021). Micronuclei are cytoplasmic masses of chromatin which are not integrated in the daughter nuclei during mitosis and that remain in the cytoplasm after cell division. The presence of micronuclei is an indicator of chromatin breakage which may be caused by clastogens or spindle dysfunctions, ultimately caused by toxic compounds (Burgeot *et al*., 1996). The MN assay, originally developed with mammalian species, is today widely applied in fish and other aquatic organisms, including sea urchin, mussels, oysters, crabs and worms, and in wild and transplanted animals.

Acetylcholinesterase (AChE) is an enzyme involved in nerve impulse transmission and its inhibition is widely used as a biomarker of neurotoxicity. Neutoroxic compounds such as organophospate and carbamate pesticides specifically inhibit AChE (Fulton and Key, 2001), but since other chemicals can also inhibit AChE it is suggested as a potential biomarker for detecting general stress in aquatic organisms. According to Prüst *et al*., 2020 AChE activity can be influenced by exposure to MPs.

"Stress in stress", a biomarker that measures air survival of mussels, provides evidence of the effects of pollutants at the whole organism level. Stress is usually defined as a measurable alteration of biochemical and/or physiological parameters induced by a change in the environment which results in a reduced capacity to adapt to further environmental challenges. Therefore, the time of mussel survival in air should be thought as a "stress on stress" response. This biomarker can be employed to assess the effects of environmental stressors at the organism level to clearly determine whether contaminants have affected the capacity of molluscs to survive further environmental change (e.g., air exposure). The "stress on stress" process is extremely simple and inexpensive (Viarengo *et al*., 2007).

The aim of the present study is to investigate the impact of MPs on mussels *Mytilus galloprovinciallis* from Greek waters. Specifically, mussels caged at five coastal areas in the Aegean (Agios Georgios, Santorini) and Ionian Sea (Strofades, Zakinthos, Gavdos) were employed in order to evaluate MP ingestion and its potential effects on mussels' physiology using biomarkers. Stress on stress, acetylcholinesterase (AchE), catalase (CAT), glutathione S-transferases (GST), lipid peroxidation (LPO) and the micronucleus (MN) test were the biomarkers used for the study.

2. Materials and Methods

2.1. Study areas and sampling

Approximately 300 mussels were sampled offshore Strofades, Laganas Bay, Gavdos, Santorini, Agios Georgios (50-60 mussels from each station) in the framework of the national MSFD monitoring in Greece between November- December 2022. The mussels had been transplanted at the sampling areas in July 2022. Particularly, mussels of approximately 60 mm were collected from a mussel farm in NW Saronikos Gulf and placed in cages (approximately 150 mussels per cage). The transplantation was performed by the Greek oceanographic vessel 'Philia'. During transplantation the mussel cages were kept on board in tanks provided with flowing seawater. The depth of the selected sites was between 20 to 30 m. The mussel cages were immersed with a buoy at approximately 5 m depth below the surface and were attached by a rope to a ballast of approximately 30 kg at the bottom. Samples were conditioned immediately after collection on board (Tsangaris *et al*., 2010).

The sites are considered as "clean" or "control sites" in terms of chemical pollution as they are far from known sources of chemical contaminants and they have been chosen in order to determine possible effects of microplastic ingestion in mussels' physiology by applying specific biomarkers. Since biomarkers also respond to chemical contaminants, these sites were chosen to avoid bias of chemical contaminants effects on biomarker levels.

One batch of mussels were used for measurements of "stress on stress" and MPs. For measurements of "stress on stress" the mussels (21-29 individuals per station) were placed in plastic boxes containing moisturized filtered paper. 13 boxes were used containing 10 mussels each and placed in an incubating chamber of 18^{o}C temperature and 12/12 h light/dark cycle. Every single box was being checked daily and in case of mortality the date was kept in order to calculate the median survival time (LT50; the time (days) when 50% of mussels are dead) and dead mussels were transferred in the freezer. These mussels were used for MPs analysis.

In another batch of musses, gills and digestive glands were dissected out immediately after collection. Pooled samples from 6 individuals (5 samples per station) were frozen and stored in liquid nitrogen for AChE, CAT, GST, LPO and protein measurements. When transferred to the laboratory samples remained at −80 °C. Few milliliters of hemolymph were smeared on slides for micronuclei assay.

2.2. Laboratory experiment

2.2.1 Experiment design to test microplastic ingestion in mussels after measurement of "stress on stress."

Before start checking microplastic ingestion in the mussels collected from the field there had to be ensured that mussels don't egest any microplastic they had taken up from their environment during measurement of "stress on stress". To understand that several mussels had to be put under the same environmental conditions with the presence of a certain number of MPs. For this reason, 30 specimens of *M. galloprocincialis* (6±1 cm shell length) were bought in January 2023 from a local mussel farm close to Athens and acclimated for five days in laboratory conditions in a big tank with artificial well-aerated sea water at salinity of 38±1 psu and temperature of 18±1°C. After acclimatization 10 mussels were chosen, cleaned and placed in 10 different plastic beakers. To minimize the risk of airborne contamination, all equipment was thoroughly washed before use with purified water (Milli-Q) and surfaces were deeply cleaned. 1L of the artificial sea water was transferred into each beaker and MPs (polyethylene) of 10mg/L in a size of 60-100μm were added in the beakers. To ensure that MPs were between 60 to 100 μm a stereoscope OLYMPUS SZX10 with an adapted camera Infinity was used and the largest dimension of each fragment was measured in μm. Every single beaker had the same number of MPs and water was well-aerated to ensure good circulation and MPs did not remain on the surface of the water. After one week of experimental exposure only 3 mussels survived, and these were placed in 3 plastic boxes and transferred in an incubation chamber with stable temperature18°C as for the measurement of "stress on stress". After the mortality of all 3 mussels, they were stored in the freezer until dissection. Mussel's length and width were measured (Table.) and soft tissue was used for microplastic analyses as described in paragraph 2.3 according to Tsangaris *et al*., 2021. The filtered paper from the plastic boxes was also kept for stereoscopic analysis in case of any microplastic egestion during "stress on stress".

2.3. Ingested MP analyses in mussels from the field

2.3.1 Microplastic extraction with KOH digestion

Mussel's length and width was measured and soft tissue was dissected out and placed into glass beakers. Strong base was used to remove biological material by hydrolyzing chemical bonds and denaturing proteins. Microplastic extraction with KOH digestion was conducted according to Tsangaris *et al*., 2021 with minor modifications. In each sample, 5 ml of 10% KOH per 1 g of wet weight of tissue was added (1:5 w/v). Samples were covered with aluminum foil and heated on thermostatic water bath (50 ◦C) until all organic matter was removed and KOH was evaporated (maximum 7 days, 5-hour

heating daily). (12h). After the digestion of the organic matter, samples were then diluted with 100 ml of purified water (Milli-Q), stirred and faaailtered under vacuum on fiberglass filters (Whatman GF/C, pore size 1.2 or 1.6 μm). Filtered samples were covered with aluminum foil and filters were placed in aluminum foil-covered Petri dishes and dried at room temperature. All filtering procedures took place inside a laminar flow cabinet.

2.3.2 Microplastic quantification

Filters were examined under a stereomicroscope OLYMPUS SZX10 using a digital camera (Luminera) and the INFINITY ANALYZE software. MP items were photographed, counted and categorized according to maximum length, color, and type (fragment, fiber, sphere), following guidelines produced by the MSFD technical group on marine litter and their largest dimension was recorded in μm.

2.3.3 Quality assurance

In the laboratory, forensic techniques, good laboratory practice and common sense should be applied to mitigate contamination. All glassware was rinsed thoroughly with purified water. Cotton lab coats were used during all laboratory procedures and the presence of staff in the lab was kept minimum. Working surfaces were cleaned with ethanol. Tissue samples were covered with aluminum foil paper during digestion and when not in use. A laminar flow cabinet was used as the working area for sample rinsing and filtration. Filters were covered with glass lids during observation under a stereomicroscope. Procedural blank samples to quantify airborne contamination were used.

2.3.4 Acetylcholinesterase (AChE)

Pooled gill tissue from 6 individuals was homogenized using a Potter-Elvehjem homogenizer (Heidolph Electro GmbH, Kelheim, Germany) in 1:2 (w:v) 0.1 M Tris–HCl buffer containing 0.1% Triton X-100, pH 7. Homogenates were centrifuged at 10,000 g for 20 min. All preparation procedures were carried out at 4 °C. AchE activity was assayed by the method of Ellman (Ellman *et al*., 1961) adapted to microplate reading by Bocquené *et al.* (1993) on a Synergy HTX, (BIOTEK) microplate reader. In each well of a microplate 60μm of sample supernatant was added and 290μm of 0.1M Tris-HCL buffer containing 0.1% TRITON 100, pH 7 plus 20μl of 0.01M DTNB (*SIGMA*) and the reaction initiated by 10μl of 0.1 acetylthiochline substrate (ACTC *SIGMA*, lot #BCBJ7056V). DTNB was being prepared daily in 0.1M pH 8 TRIS/HCL and ACTC in distilled water was also being prepared fresh daily. The enzyme kinetic was read every 15s for 2 min at 414nm and every sample was measured in triplicate. Specific enzyme activity was expressed as nmoles ACTC/min/mg protein.

2.3.5 Catalase (CAT) activity

Digestive glands were homogenized using a Potter-Elvehjem homogenizer in 1:4 (w/v) 100 mM KH_2PO_4/K_2HPO_4, pH 7.4. Homogenates were centrifuged at 10,000 g for 30 min. All preparation procedures were carried out at 4 °C. CAT activity was measured by the method of Cohen *et al*. (1996) by the loss of hydrogen peroxide ($H_2\ O_2$)after incubation with the enzyme. Ferrus ions and thiocyanate are used for the colorimetrical determination of $H_2\ O_2$ .In particular, in 5ml tubes 850μl phosphate buffer was added, 50 μl sample and 100 μl of H_2O_2 60 mM. At 1 and 4 minutes after initiation of reaction 100 μl aliquots were removed and added to the mixture of 4.0ml of 0.6 N H2SO4 plus 1.0ml of 10 mM FeSO4 at room temperature and tubes were covered with aluminium foil. The red color of ferrithiocyanate was measured at 490nm on the microplate reader at room temperature. Duplicate readings were taken for every single tube.CAT activity results are expressed in terms of the first order reaction rate constant (k) and protein content as follows: U/mg proteins=k/mg proteins=[ln (A1/A2)/ (t_2-t_1)]/mg proteins where ln is the natural log, A1 and A2 are the observed mean absorbance at 490 nm at two time points, t_1=1 min and t_2=4 min.

2.3.6 Glutathione S-transferases (GST) activity

Preparation of digestive gland tissue extracts were as described for CAT. The GST activity was evaluated following the conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) as described in Habig et al (Habig *et al*., 1974). Specifically, 0.2M phosphate buffer pH 6.5, 1mM CDNB, 1MmGSH was used as reaction mixture. In each well of the microplate, 15μl of sample in triplicate was added with 200μl of the mixture. The absorbance was measured at 340 nm for 4 min at 25°C and expressed as nmol CDNB conjugate formed/min/mg protein.

2.3.7 Lipid peroxidation (LPO) activity

Preparation of digestive gland tissue extracts was as described for CAT. The LPO was determined as thiobarbituric acid reactive substances (TBARS) following the procedure of S and Bird and Draper (Bird & Draper, 1984) with some modifications. Specifically, butylated hydroxytoluene (BHT 4%) was added in 50 μl of sample, followed by 500 μl trichloroacetic acid (12%), 450 μl phosphate buffer and 500 μl 2-thiobarbituric acid (0.73%). The samples were incubated at 100 °C for 1 h and then centrifuged at 13400g for 5 min in 4oC. The absorbance of the supernatant was measured using a spectrophotometer plate reader at 535 nm and the LPO was expressed as nmol TBARS formed per mg protein using the molar absorption coefficient for TBARS (ε = 1.56 x 10^{-5} M^{-1} cm^{-1}).

2.3.8 Total protein concentration

Total protein content in the homogenate supernatants was measured by the Bradford method (1976) adapted for microplate reading. Protein concentration was calculated using a Bovine Serum Albumin (BSA) standard curve of 0.1 mg/ml solution in distilled water. In each microplate well 100µl of diluted sample was added and 280 µl of Bradford reagent (1/5 water diluted Bio-Rad Bradfords reagent) and the absorbance was read at 595nm.

2.3.9 Micronucleus frequency measurements (MN test)

The micronuclei (MN) frequency was assessed in order to evaluate micronuclei formation in mussel haemolymph as a genotoxic biomarker. The test was performed using the method of Galloway *et al*. (2010) with a few modifications. 10 mussels were used for the MN test. From those mussels few milliliters of haemolymph were smeared on clean glass slides. The slides were air dried for 30 min, fixed in ethanol for 10 min and stained with 5% Giemsa solution in 0.1 M phosphate buffer for 15 min. The slides were washed twice with phosphate buffer and let dry. The stained slides were analyzed under light microscope at a final magnification of x1000. For each specimen, minimum 400 cells with intact cytoplasm were scored. The MN was identified according to the following criteria:

1. Spherical or ovoid-shaped extra nuclear bodies in cytoplasm
2. A diameter of 1/3 of the main nucleus
3. Non-refractory bodies
4. Colour, texture and optical features resembling those of the nucleus.
5. Bodies completely separated from the main nucleus.

The boundary of a MN should be intact and completely distinguishable from the boundary of the main nucleus, otherwise it cannot be considered as MN. Only the clearly isolated MN (< 1/3 of the nucleus) were counted as such and other nuclear anomalies (e.g., nucleus buds) were recorded separately.

The frequency of micronucleus (MN) is expressed as the number of MN per 1000 cells.

2.3.10 Statistical analysis

MPs and biomarkers data were checked for normality using the Shapiro-Wilk test. MP data did not comply with the assumption of normality (Shapiro-Wilk test), thus the Kruskal-Wallis test was conducted to evaluate differences in the number and size of ingested MPs among sampling sites AChE, CAT, GST and LPO data were normally distributed (Shapiro-Wilk test) and were analyzed by one-way ANOVA. Post hoc test for multiple comparisons was used to test any differences in biomarkers between sites. MN frequencies were not normally distributed and thus the Kruskal-Wallis test was applied to test for differences between sites. Spearman's correlation was used to evaluate relations between number of ingested MPs and biomarkers values. Relations between shell length of mussels and MP size were tested using Kruskal-Wallis test and Spearman's correlation for non-parametric data. Significance for all statistical tests was established at $p < 0.05$. Statistical analyses were performed using SPSS Statistics 17.0. The graphs and tables were created using Microsoft Excel.

3. Results

3.1 Microplastic ingestion

In total, 129 mussels sampled from Greek coastal waters were studied for microplastic ingestion. The mean (± SE) length and width of the mussels was 6.37±0.18 and 3.38±0.09 cm respectively and their mean (±SE) weight (soft tissue) was 4.09±0.69 g. Mean length, width and weight of mussels at each station are given in Table 1. Furthermore, MP ingestion was examined in the 3 mussels, exposed to MPs in laboratory conditions and filtered papers where the mussels were placed on were checked for any egested MPs during "stress on stress."

3.1.1 MP quantification

Quantitative results of MPs detected in the Mediterranean mussels for each sampling station are given in Table 1 after blank values were subtracted from the final data (i.e. items similar to those found in the blanks excluded from the results). The total number of MPs found in mussels at all sites was 126 items. The highest MP number was found to be at the station Aghios Georgios with 31 MPs in total, whereas the lowest number was found to be in station Santorini with 17 MPs in total.

The total of 126 MPs was observed in 84 mussels collected from all sampling sites. By proportional calculation, the frequency of occurrence of mussels with ingested MPs was 65.12% and the MP abundance in all mussels examined (mean number of MPs/individual) was 0.96 ±0.18 (Table 1). Considering only positive samples from all sites, mean MP abundance was 1.51±0.18 items/individual.

Although Aghios Georgios was the station with the highest MP number (31 items) the number of mussels containing MPs (positive samples) was 20 compared to Strofades having less items in total (29) but same number of mussels containing them (Table 1). Finally, Santorini, Zakynthos and Gavdos had 12,18 and 14 positive samples, respectively, while 17, 28 and 21 items were detected in each station. Frequency of MP occurrence ranged from 57.14% at Santorini to 74.07% at Agios Georgios (Table 1). It seems that these results do not show a high spatial variation and the frequency of occurrence of mussels with ingested MPs is over 50% for all sites.

Average number of MPs per individual ranged from 0.81 at Santorini to 1.15 at Agios Georgios and showed no significant differences among sites (Kruskal-Wallis test,

p=0.264 >0.05) (Table 2a). Average number of MPs per individual in positive samples was 1.55 ±0.18 in Agios Georgios, 1.56±0.17 in Zakynthos, 1.53± 0.11 in Strofades, 1.5±017 in Gavdos and 1.42± 0.26 in Santorini (Table 1) and showed no significant differences among sites with p=0,831 (Kruskal-Wallis, p>0.05). (Table 2b).
As for the 3 mussels exposed to MPs in laboratory conditions, many of the MPs that had been added in the water were recognized in their digested tissues filtrates meaning that mussels had successfully consumed and ingested some of these MPs.
Furthermore, the filter paper where the mussels were placed on after MP exposure was checked carefully, confirming that none of the mussels that were fed with MPs in laboratory conditions had egested any of those particles during exposure to air for measurement of "stress on stress".

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SITE	Strofades	Gavdos	Santorini	Agios Geor	Zakynthos	ALL STATIONS
Number of individuals examined	29	24	21	27	28	129
Number of individuals containing microplastics	20	14	12	20	18	84
MP frequency of occurrence	68.97%	58.33%	57.14%	74.07%	64.29%	65.12%
MP number	29	21	17	31	28	126
MP abundance						
a) Number of items per individual in all individuals examined	1±0.16	0.83±0.17	0.81±0.21	1.15±0.19	1±0.18	0.96±0.18
b) Number of items per individual in individuals containing microplastics	1.53±0.11	1.5±0.17	1.42±0.26	1.55±0.18	1.56±0.17	1.51±0.18
Average number and standard deviation of						
a) mussel's length	6.5±0.61	6.3±0.42	6.59±0.57	6.14±0.54	6.34±0.43	6.4±0.52
b) mussel's width	3.48±0.35	3.44±0.20	3.42±0.35	3.29±0.30	3.29±0.27	3.4±0.3
c) mussel's soft tissue weight	4.83± 1.95	3.85±1.34	4.74±1.94	3.92±1.30	3.15±1.08	4.0±1.5

Table 1*: Number of individuals examined from each site, number of individuals containing MPs, MP frequency of occurrence (%), MP number from each station and MP abundance in a) all individuals and in b) individuals containing MPs and average number ± standard deviation of mussel's length,width and soft tissue weight*

3.1.2 MP characterization (shape, size, color)

The most abundant color of the MPs was blue with a percentage of 58.73% followed by the transparent items 15.08% and white 7.94% (Figure 4). The other colors were in much less numbers and are also shown in Figure 4.
All the detected colors were the blue, black, red, green, transparent, grey, white and purple. The last one was only found once in Strofades station. In total, there were found 74 blue items,19 transparent,10 white, 8 black 6 red, 5 green, 3 grey and 1 purple.

The distribution of the shapes in relation to the size revealed that the larger particles (>1 mm) were exclusively represented by fibers, while smaller size classes (< 1mm) were mostly formed by fragments and films. Fibers were the most abundant MP shape category in all sites with the following percentages: Zakynthos 67.86%, Agios Georgios 54.84%, Strofades 55.17%, Gavdos 66.67% and Santorini 70.59%. The percentage of fragments was 41.94% in Agios Georgios, 41.38% in Strofades, 28.57% in Zakynthos, 33.33% in Gavdos and 29.41% in Santorini. Furthermore, films constitute only the percentage of 3.45% in Strofades. 3.57% in Zakynthos and 3.23% in Agios Georgios (Figure 4).

MPs were classified in 4 different size categories. First category particles were between 0.1 to 0.49mm (1), the second category 0.5-1.0mm (2), the third category 1.01-5.0mm (3) and the last category included MPs larger than 5mm particles (4). In category number 1 the percentages are the following: Strofades 52%, Gavdos 19%, Santorini 41%, Agios Georgios 52% and Zakynthos 36%. For category number 2 the results are: Strofades 17%, Gavdos 33%, Santorini 41%, Agios Georgios 10% and Zakynthos 36%. Category number 3 contains the following results: Strofades 31%, Gavdos 38%, Santorini 18%, Agios Georgios 39% and Zakynthos 29%. In the last category only 10% of items from station Gavdos were classified as more than 5. 0mm. In total 52 of the 126 items found in mussels were between 0.1-0.49mm in size followed by 40 items range between 1.01-5mm, 32 items 0.5-1.0mm and only 2 of them being more than 5 mm. Furthermore, Kruskal-Wallis test showed that the distribution of mussel's length across all sites is the same as it is for MPs size as well (P=0.409 and P=0.822 respectively). Spearman's correlation between mussel's shell length and MP's size showed no significant correlation (P=0.123) (Table 3)

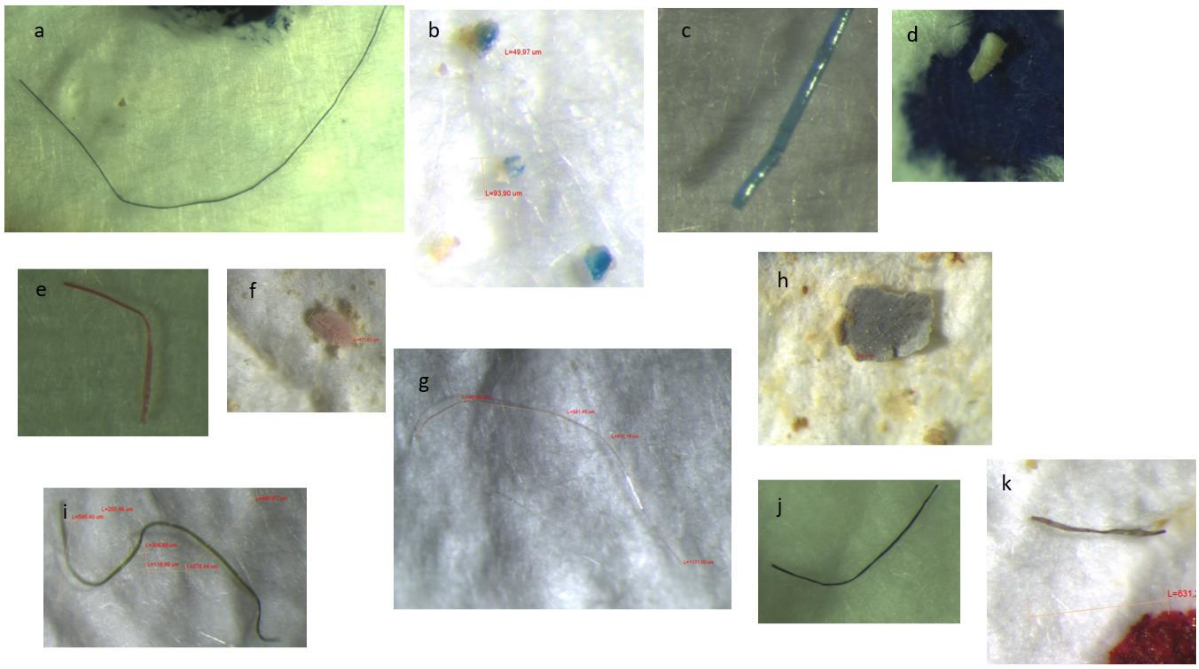

Figure 1*: Microplastics found in mussel's tissue: a) Blue fiber b) blue fragments c) blue fragment d) white fragment e) red fiber f) red fragment g) transparent fiber h) grey film i) green fiber j) black fiber k) purple fiber.*

a

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of MP_possitive_samples is the same across categories of SITE_4.	Independent-Samples Kruskal-Wallis Test	,831	Retain the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

b

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution MPs is the same across categories of SITE.	Independent-Samples Kruskal-Wallis Test	,264	Retain the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

Table 2*: Kruskal-Wallis test shows no significant differences among sites between a) all individuals b) positive individuals*

Correlations

			mussel_length	mp_size
Spearman's rho	mussel_length	Correlation Coefficient	1,000	-,137
		Sig. (2-tailed)	.	,123
		N	129	129
	mp_size	Correlation Coefficient	-,137	1,000
		Sig. (2-tailed)	,123	.
		N	129	129

Table 3*: Spearman's correlation between mussel's length and MP size*

Correlations

			lt50	mplastics
Spearman's rho	lt50	Correlation Coefficient	1,000	,105
		Sig. (2-tailed)	.	,866
		N	5	5
	mplastics	Correlation Coefficient	,105	1,000
		Sig. (2-tailed)	,866	.
		N	5	5

Table 4: *: Spearman's correlation between LT50 and mean number of MPs*

Correlations

			average_mps	AVERAGE_ACHE	AVERAGE_CAT	AVERAGE_GST	AVERAGE_LPO
Spearman's rho	average_mps	Correlation Coefficient	1,000	-,564	-,564	-,205	,051
		Sig. (2-tailed)	.	,322	,322	,741	,935
		N	5	5	5	5	5
	AVERAGE_ACHE	Correlation Coefficient	-,564	1,000	,100	,500	-,300
		Sig. (2-tailed)	,322	.	,873	,391	,624
		N	5	5	5	5	5
	AVERAGE_CAT	Correlation Coefficient	-,564	,100	1,000	-,100	-,600
		Sig. (2-tailed)	,322	,873	.	,873	,285
		N	5	5	5	5	5
	AVERAGE_GST	Correlation Coefficient	-,205	,500	-,100	1,000	,300
		Sig. (2-tailed)	,741	,391	,873	.	,624
		N	5	5	5	5	5
	AVERAGE_LPO	Correlation Coefficient	,051	-,300	-,600	,300	1,000
		Sig. (2-tailed)	,935	,624	,285	,624	.
		N	5	5	5	5	5

Table 5: *Spearman's correlation between MPs and biomarkers (AChE,CAT,GST,LPO)*

Correlations

			aver_MN	average_mps
Spearman's rho	aver_MN	Correlation Coefficient	1,000	,132
		Sig. (2-tailed)	.	,833
		N	5	5
	average_mps	Correlation Coefficient	,132	1,000
		Sig. (2-tailed)	,833	.
		N	5	5

Table 6: *Spearman's correlation between MPs and MN*

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
AChE	Between Groups	2023,924	13	155,686	,576	,829
	Within Groups	2975,370	11	270,488		
	Total	4999,294	24			
GST	Between Groups	896,711	13	68,978	1,062	,466
	Within Groups	714,579	11	64,962		
	Total	1611,290	24			
CAT	Between Groups	15,029	13	1,156	1,031	,486
	Within Groups	12,332	11	1,121		
	Total	27,361	24			
LPO	Between Groups	17,684	13	1,360	,420	,930
	Within Groups	35,621	11	3,238		
	Total	53,305	24			

Table 7*: One-way ANOVA test between biomarker levels (AChE,CAT,GST,LPO) and sites*

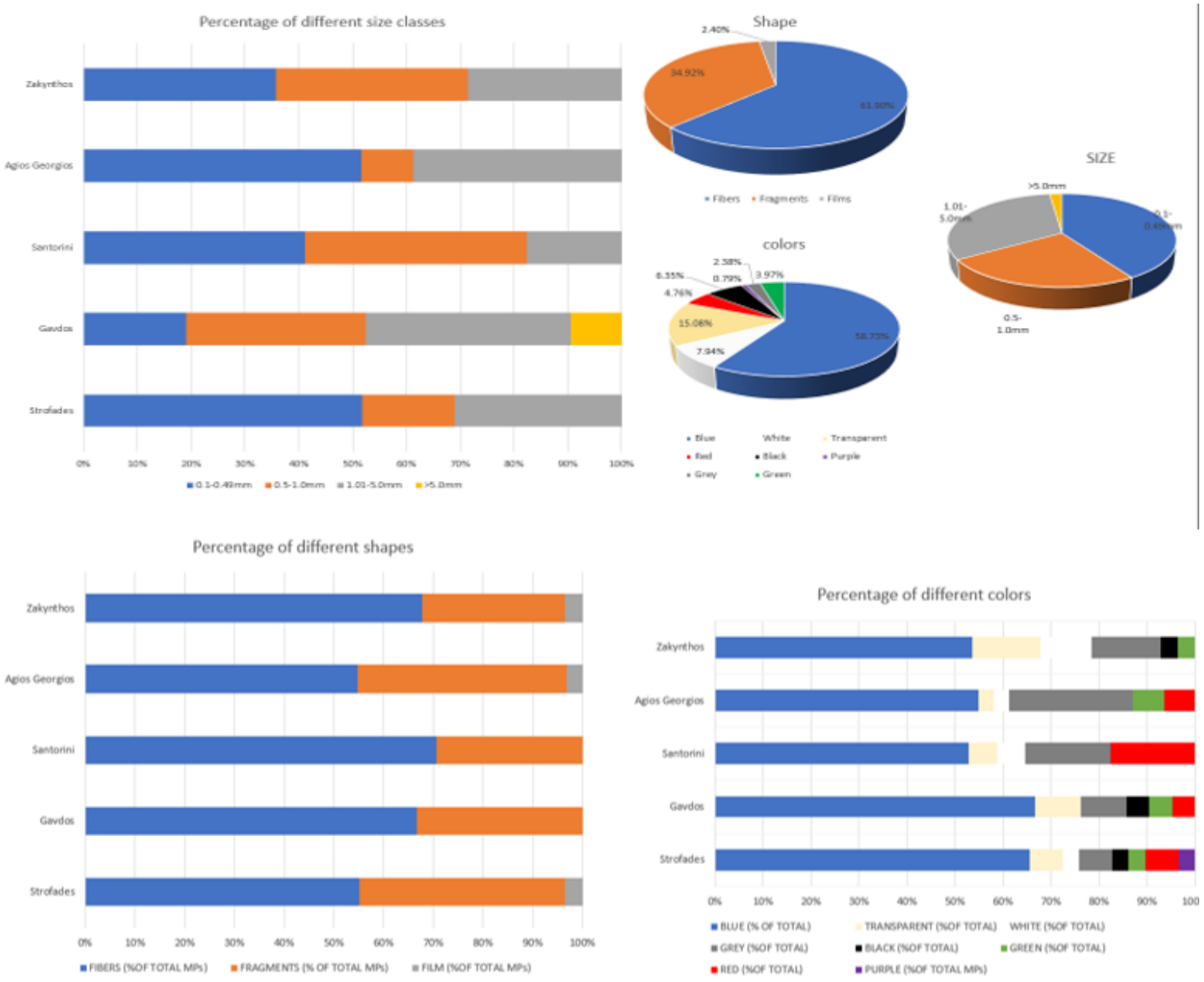

Figure 4*: Shape, size and color of MPs detected in mussels (Mytilus galloprovincialis) sampled from Strofades, Gavdos, Agios Georgios, Zakynthos and Santorini.*

3.3 Biomarkers (Stress on stress, AChE, GST, CAT, LPO, MN) responses

"Stress on stress" values were calculated as LT50 which indicate the time at which 50% of the air exposed organisms die. LT50 was lowest at Zakynthos and highest at Agios Georgios (9±0.4 days at Agios Georgios, 6±0.4 days at Zakynthos, 7±0.9 days at Strofades, 8±0.6 days at Santorini and 8±0.7 days at Gavdos) and were not related to average MP abundance Table 4 (Spearman's correlation, $p>0.05$).

Mussels from Strofades showed highest average AChE activity (44.3 nmoles ACTC/mg protein), while the lowest level was in mussels from Zakynthos (27.2 nmoles ACTC/mg protein). The average GST activity was highest in samples analyzed from Santorini (37.6 nmoles CDNB/mg protein) and lowest in samples taken from Zakynthos (23.1 nmoles CDNB/mg protein). The highest average value of CAT was measured in samples from Gavdos (3.71 units/mg protein) and the lowest in samples from Zakynthos (1.48 units/mg protein). Finally, LPO levels found much higher in samples from Zakynthos (4.51 nmolesTBARS/mg protein) compared to the others. (Figure 2). However, differences in AChE, CAT, GST activities and LPO among sites were not significant (One-way Anova, $p>0.05$) (Table 7).

Spearman's correlation between average microplastic abundance in each site and average AChE , CAT, GST activities as well as LPO levels showed no significant relations ($P>0.05$) (Table 5).

As regards the MN test, according to the Galloway *et al*., 2010 protocol, a total of 1000 cells per mussel should be analyzed in order to have accurate results. In our study, since 1000 cells could not be counted in every single sample, MN frequency was calculated by the number of MN counted per 400 or more cells scored. So, from the ten mussels of each station that were being checked for MN, about 2 to 5 could finally, be used for the analysis because for the rest of them less than 400 cells were counted. At least five different nuclear abnormalities were recognized in mussel's heamocytes and counted for every single sample of each station. Main nuclear abnormalities were MN, binucleated (BN), nucleus bud, two-lobed and eight shaped shown in figure 3. Results showed that the highest MN frequency being 3.8 per 1000 cells found in a mussel from Agios Georgios station having 2 MN out of 527 cells. Considering averages MN frequencies, Gavdos was the station with the highest average MN frequency being 1.9±0.06 and Zakynthos was the station with the lowest average MN frequency being 0.7±0.7. Statistical analysis showed that MN frequencies are not normally distributed and Kruskal-Wallis test showed that the distribution of MN frequencies is the same among sites and the median MN frequency observed in the present study is close to 0. Spearmen's correlation between MP abundance and mean MN frequency showed no significant difference ($P=0.833$) (Table 6).

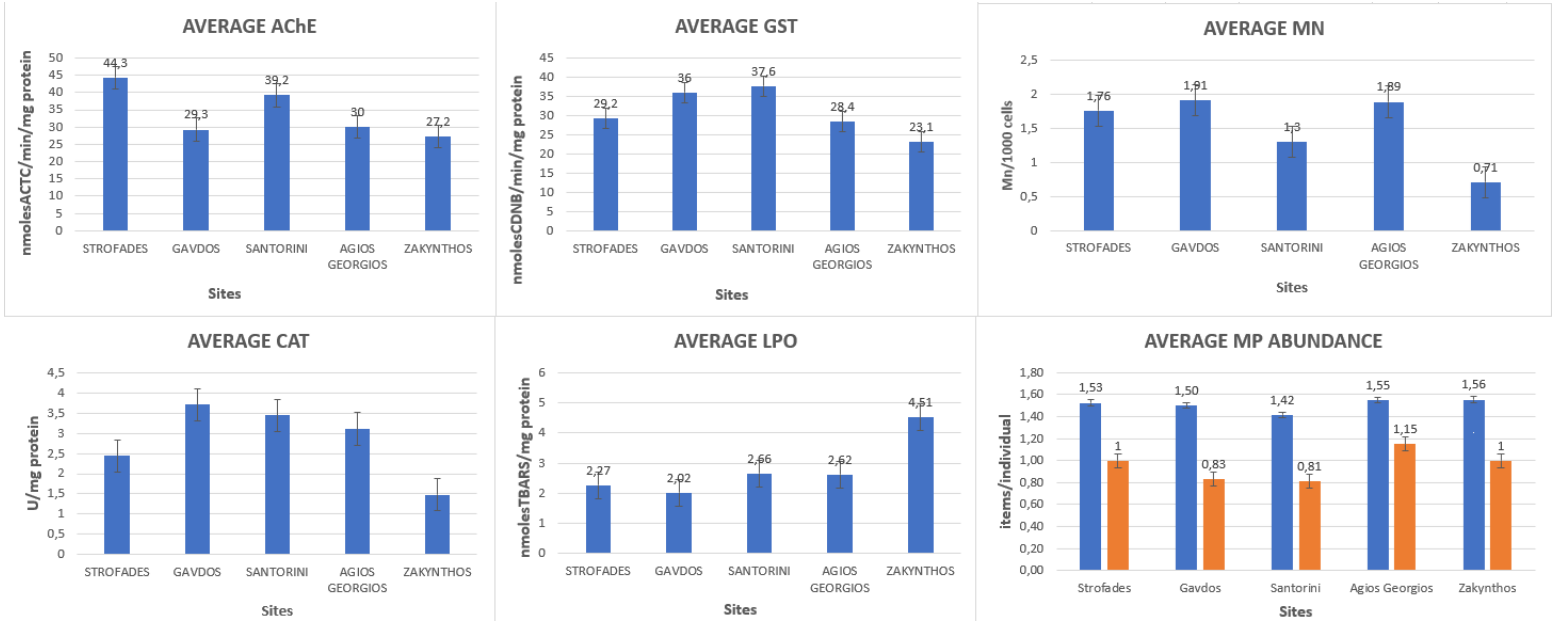

***Figure 2**: Average activities of AChE, GST and CAT, levels of LPO and MN abundance measured in mussels from each station.*

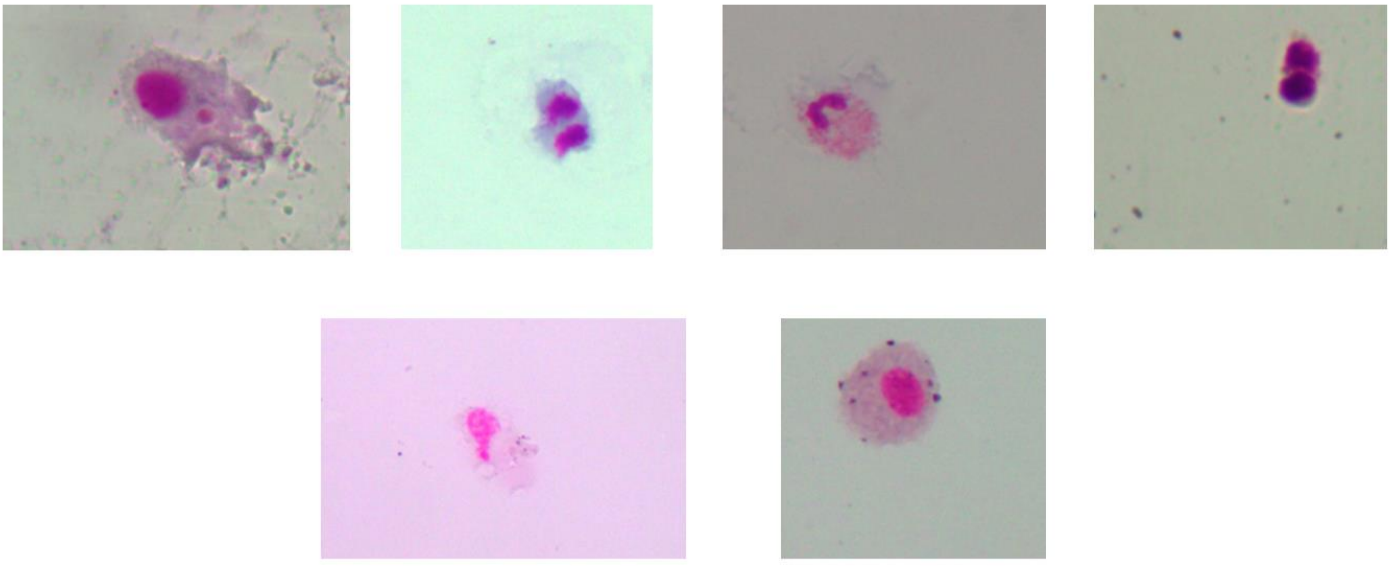

***Figure 3**: 5 different nuclear abnormalities found in mussel's hemolymph: 1) MN 2)BN 3)two-lobed 4) eight-shaped 5) nucleus bud 6) normal cell*

4. Discussion

This study provides an evaluation of MP contamination in mussels *Mytilus galloprovincialis* from Greek waters. As they are filter-feeding organisms they tend to filter great amounts of water and ingest MPs, that can lead to physical harm and physiology changes.
Specifically, mussel samples from Greek waters far from known chemical pollution sources; Strofades, Agios Georgios, Zakynthos, Gavdos and Santorini were collected in order to evaluate MP ingestion and in parallel specific biomarkers were applied in order to identify possible effects of MP ingestion on mussels' physiology. Stress on stress, acetylcholinesterase (AChE) in gill tissue, catalase (CAT), glutathione S-transferases (GST) and lipid peroxidation (LPO) in the digestive gland of the mussels and micronuclei test in the hemolymph were the biomarkers used in the present study. Our findings provide further evidence that mussels are prone to MP ingestion (frequency of occurrence 65.12%) as documented in the Greek areas of the present study.

4.1 Microplastic ingestion

A total of 126 microparticles were visually identified as MPs as a result of stereoscopic observation after the separation of the particles that were also found in the blank samples.
MP abundance was expressed for each area in 2 different ways; a) average number of MP items per individual in all individuals examined b) average number of MP items in positive samples. Frequency of occurrence of ingested items was calculated as the percentage of the individuals examined containing MPs.
MP abundance (Table 1) among all mussel samples (0.96±0.18) and among positive samples (1.51±0.18) of all the areas examined was compared with other studies conducted in different sites (Table 8). Similar values were reported from samples collected from Northern Ionian Sea (0.9±0.2 items/individual in all individuals and 1.9±0.2 items/individual in positive samples (Digka *et al*., 2018). In this study fibers were found to be more abundant, thus in the study conducted in mussels from Northern Ionian Sea fragments were the most abundant microplastic shape category (Digka *et al*., 2018). Results of a study conducted in Adriatic Sea showed that the number of ingested particles in caged mussels roughly varied from 1 to 2 items/individual with fibers occupying the percentage of 62% (Avio *et al*., 2017). Lower values (0.76 ± 0.40) were found for mussels analyzed from Baie des Veys along French coast with fibers being the main plastic particle (Hermabessiere *et al*., 2019). Furthermore, similar values (0.69 items/individual) were found in mussels analyzed from Turkish coasts, with fragments being the most abundant shape (67.6%) (Gedik & Eryaşar, 2020). A study conducted in Estuary of Forth River Edinburgh, UK showed that fibers were the most abundant shape and mean abundance was 10.4 ± 3.42 possibly because of airborne contamination. Higher values were also found to other non-anthropogenic impacted areas like locality of Andratx (southwest of Mallorca Island) (4.54 ± 0.34 items/individual) (Capo *et al.*, 2021).

Species	No of individuals	frequency of occurrence	Mean MP abundance per individual	Mean MP abundance per individual in possitive samples	Abundant shape	Area	References
M. galloprovinci	129	65,12%	0.96±0.18	1.51±0.18	fibers	greek waters (control areas)	this study
M. galloprovinci	80	46,30%	0.9 ± 0.2	2.0 ± 0.2	fragments	northern ionian coast	Digka *et al*., 2018
caged mussels	30	36%		1.54 ± 0.93	fibers	Adriatic sea (control area)	Avio *et al*., 2017
Mytilus galloprov	25	-	0.69	-	fragments	Turkey	Gedik & Eryaşar, 2020
Mytilus sp.	8	-	10.4 ± 3.42	-	fibers	Estuary of Forth River Edinburgh, UK	Catarino et al., 2016
Mytilus galloprov	24	-	4.54 ± 0.34	-	-	Locality of Andratx (southwest of Mallorca Island)	Capo *et al*., 2021
Mytilus edulis	50	-	0.76 ± 0.40	-	fibers	Baie des Veys	Hermabessiere *et al*., 2019

***Table 8**: Microplastic ingestion reported in the literature for Mytilus sp.*

Since the mussels used to evaluate MP ingestion in the present study were those used for measurement of "stress on stress", to validate MP ingestion results, a laboratory experiment was conducted in order to test if MPs are egested by the mussels during "stress on stress" measurements. The mussels exposed to MPs in the laboratory that were then air exposed and checked for MP egestion did not confirm the suspicion that maybe they reject some of the ingested particles during exposure to air for measurement of "stress on stress". Although further study must be done and more mussels need to be checked under laboratory conditions if we want to accept this fact.
In our study, frequency of occurrence seems to be much higher compared to other studies conducted recently (Digka *et al.* 2018, Avio *et al*. 2017). It should be taken into consideration that FTIR analyses was not used in order to confirm which of the particles were MPs.

4.2 Biomarkers

Biomarkers are a variety of biological indicators that are measurable quantitatively and indicate changes at the cellular, biochemical, molecular, and physiological levels (Lionetto *et al*., 2019). These indicators can be measured at the cellular level, body fluid level, tissue level or organ level. Biomarker results of the present study did not show responses that can be attributed to the presence of MPs as there were no significant differences in numbers of ingested MPs or biomarkers among sites and no significant correlations of ingested MPs and biomarkers.
Stress on stress values in mussels at all sites were higher than established environmental assessment criteria - EAC (LT50=5 days) but lower than background assessment

criteria-BAC (LT50=10 days) (ICES, 2019), suggesting that the mussels were not severely stressed but also not in a "very good" health condition. This may be due to environmental factors, such as high temperature, and gonadal development that can also contribute to lower survival time of mussels.

In mussels it has been evidenced that exposure to pollutants like heavy metals and organic contaminants as well as MPs causes an increase in GST, LPO and CAT activity (Vidal-Liñán *et al*., 2010, Sureda *et al*., 2011, 2018; Capo e*t al.,* 2015b, Capo *et al*., 2021). In the study by Capo *et al*., 2021, mussels analyzed from aquaculture cages had increased GST activity values which coincided with a higher intake of MPs. In the present study, GST activities ranged between 13.3-50.6 nmol/min/mg protein showing higher mean values in Santorini station and Gavdos (Figure 2) which were the sites with the lower MP abundance in positive samples in contrast to the results of Capo et al, 2021. Low GST enzyme activities have been also reported in Mytilus spp., in response to pollutants (Fernández *et al*., 2012) and decreased activity was measured in the digestive gland and gills of *M. edulis* in the presence of PE and PP plastic particles (Revel *et al*., 2019）. Many studies have shown positive correlations between levels of antioxidant defenses and the presence (levels, concentration) of xenobiotics (Orbea *et al.,* 2002) so it is expected to have increased LPO and CAT levels in mussels from more contaminated places. In this study, highest CAT (3.71 units/mg protein) and LPO (4.51 nmolesTBARS/mg protein) levels were found in mussels from Gavdos and Zakynthos respectively (Figure 2). Zakynthos is the place with the highest MP abundance in positive samples while Gavdos is the third. Similar results were also obtained from mussels collected and analyzed from a farm located to a French coast, where CAT increased in organs that was measured in presence of MPs as a defense mechanism （Revel *et al*., 2019. Different responses in CAT have also been found in studies where, the activity of this enzyme found higher in reference sites, maybe because of the season that the study was conducted that seems to influence the food availability and therefore the enzyme (Nesto *et al*., 2004).

AChE activity measured in mussels, as marker of neurotoxicity has been found to decrease after organism's exposure to MPs (Iheanacho & Odo, 2020). In our study, AChE activity was found lowest in mussels from Zakynthos (Figure 2) which was the station with the highest MP abundance in positive samples and highest in sites Strofades and Santorini (44.3 and 39.2 nmoles ACTC/mg protein, respectively). Santorini is the place with the lowest MP abundance and Strofades the third. Similar responses were observed in Tsangaris et al. (2010) study where AChE activity showed lower levels in mussels from sites influences by anthropogenic factors. Highest values of AChE were also reported in mussels (M. *trossulus*) being present in favourable environmental conditions (Kopecka *et al*., 2004). Overall, US EPA (1998) suggests that a significant AChE activity decrease by 20% or more from the pre-exposure can be considered as a clear toxicological effect of xenobiotics. In our study we cannot estimate the level change as we don't have pre-exposure measurements.

Mussels at Gavdos station had the highest average MN frequency (1.9) (Figure 2) although MN results should be treated with caution since our study could not follow

the protocol of 1000 cells counts. As was mentioned above Gavdos appeared to have the highest average values in CAT (3.71 units/mg protein) and GST (36 nmol/min/mg protein) activities. Furthermore, AChE seem to have lower values in mussels from Gavdos compared to the other sites apart from Zakynthos. Gavdos was the only place where a relation between MN frequency and biomarkers of oxidative stress and neurotoxicity (CAT, GST, AChE) can be assumed, but this cannot be attributed to the presence of MPs that were not found in higher numbers in these mussels.

Statistical analysis did not show significant differences in MP ingestion among sites. Similarly, MN frequencies AChE, LPO, CAT and GST did not show significant differences among sites and could not be associated to MPs exclusively. It can be assumed that other environmental factors or biological variables (temperature, oxygen, mussel's condition, age) could have affected mussel's physiology (Bellas *et al*., 2014)**.** Mussels seem to get affected by environmental factors such as water temperature, salinity, dissolved oxygen, nutrients, and parasites, that exhibit short and long-term fluctuations (Parisi *et al*., 2017). Among abiotic factors, thermal stress can constitute a physiological disorder in an aquatic organism, which potentially affects metabolism (Lockwood *et al*. 2010). For instance, water temperature has been shown to have a direct effect on the mitotic rate and consequently on the formation of MN. (Schiedek *et al*., 2006). Other studies have shown that seasonal variation can affect biomarker activities. For example, it has been found that AChE and GST activities increase in summer and decrease in winter (Kaaya *et al.*, 1999; Vidal-Liñán & Bellas, 2013). Maybe the results of the present study relate to other environmental factors and trophic characteristics of the sites. For example, oligotrophic areas have less food for bivalves affecting their growth and finally their physiology (Tsangaris *et al*., 2010).

Conclusions

In this study, the number of MPs found in mussels tissue ranged between 0 to 4 items. Biomarker responses could not be attributed to the presence of MPs as there were no significant differences in numbers of ingested MPs or biomarkers among sites and no significant correlations of ingested MPs and biomarkers. Maybe the low number of ingested MPs was not enough to affect the specific biomarkers that were used. Furthermore, biomarkers may show biphasic responses or can be influenced by environmental factors other than pollutant exposure. More research needs to be done in order to detect any correlation between biomarkers and MPs and maybe more samples need to be examined in order to confirm these results.

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References

Abel, P. D. (1976). Effect of some pollutants on the filtration rate of Mytilus. *Marine pollution bulletin*, *7*(12), 228-231.

Agriculture Organization of the United Nations. Fisheries Department. (2018). *The state of world fisheries and aquaculture*. Food and Agriculture Organization of the United Nations.

Albano, M., Panarello, G., Di Paola, D., Capparucci, F., Crupi, R., Gugliandolo, E., ... & Savoca, S. (2021). The influence of polystyrene microspheres abundance on development and feeding behavior of Artemia salina (Linnaeus, 1758). *Applied Sciences*, *11*(8), 3352.

Allsopp, M., Walters, A., Santillo, D., & Johnston, P. (2006). Plastic debris in the world's oceans. *Greenpeace, Amsterdam*.

Andrady, A. L. (2017). The plastic in microplastics: A review. *Marine pollution bulletin*, *119*(1), 12-22.

Arthur, C., Baker, J. E., & Bamford, H. A. (2009). Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris, September 9-11, 2008, University of Washington Tacoma, Tacoma, WA, USA.

Avio, C. G., Cardelli, L. R., Gorbi, S., Pellegrini, D., & Regoli, F. (2017). Microplastics pollution after the removal of the Costa Concordia wreck: First evidences from a biomonitoring case study. *Environmental Pollution*, *227*, 207214.

Balbi, T., Camisassi, G., Montagna, M., Fabbri, R., Franzellitti, S., Carbone, C., ... & Canesi, L. (2017). Impact of cationic polystyrene nanoparticles (PS-NH2) on early embryo development of Mytilus galloprovincialis: Effects on shell formation. *Chemosphere*, *186*, 1-9.

Barnes, D. K., Galgani, F., Thompson, R. C., & Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical transactions of the royal society B: biological sciences*, *364*(1526), 1985-1998.

Bellas, J., Albentosa, M., Vidal-Liñán, L., Besada, V., Franco, M. Á., Fumega, J., ... & Beiras, R. (2014). Combined use of chemical, biochemical and physiological variables in mussels for the assessment of marine pollution along the N-NW Spanish coast. *Marine environmental research*, *96*, 105-117.

Beyer, J., Green, N. W., Brooks, S., Allan, I. J., Ruus, A., Gomes, T., ... & Schøyen, M. (2017). Blue mussels (Mytilus edulis spp.) as sentinel organisms in coastal pollution monitoring: a review. *Marine environmental research*, *130*, 338-365.

Bivalves' early evolution as revealed by mitochondrial genes. *Molecular Phylogenetics and Evolution*, *57*(2), 641-657.

Bocquené, G., & Galgani, F. (1998). *Biological effects of contaminants: cholinesterase inhibition by organophosphate and carbamate compounds*. ICES Techniques in Marine Environmental Science (TIMES).

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

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., & Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, Mytilus edulis (L.). *Environmental science & technology*, *42*(13), 50265031.

Burgeot, T., Woll, S., & Galgani, F. (1996). Evaluation of the micronucleus test on Mytilus galloprovincialis for monitoring applications along French coasts. *Marine Pollution Bulletin*, *32*(1), 39-46.

Capó, X., Company, J. J., Alomar, C., Compa, M., Sureda, A., Grau, A., ... & Deudero, S. (2021). Long-term exposure to virgin and seawater exposed microplastic enriched diet causes liver oxidative stress and inflammation in gilthead seabream Sparus aurata, Linnaeus 1758. *Science of The Total Environment*, *767*, 144976.

Capo, X., Rubio, M., Solomando, A., Alomar, C., Compa, M., Sureda, A., & Deudero, S. (2021). Microplastic intake and enzymatic responses in Mytilus

Castorani, M. C., Glud, R. N., Hasler-Sheetal, H., & Holmer, M. (2015). Light indirectly mediates bivalve habitat modification and impacts on seagrass. *Journal of Experimental Marine Biology and Ecology*, *472*, 41-53.

Chamelea gallina, from the Adriatic sea. *Food Chemistry*, *101*(3), 1063-1070.

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

Cooper, D. A. (2012). *Effects of chemical and mechanical weathering processes on the degradation of plastic debris on marine beaches*. The University of Western Ontario (Canada).

Digka, N., Tsangaris, C., Torre, M., Anastasopoulou, A., & Zeri, C. (2018). Microplastics in mussels and fish from the Northern Ionian Sea. *Marine Pollution Bulletin*, *135*, 30-40.

EPA (2013). Health Effects of PCBs. Retrieved from http://www.

epa.gov/epawaste/hazard/tsd/pcbs/pubs/effects.htm.

Europe, P. (2015). An analysis of European plastics production, demand and waste data. *Plastics–the facts*, *147*.

Farrington, J. W., Tripp, B. W., Tanabe, S., Subramanian, A., Sericano, J. L., Wade, T. L., & Knap, A. H. (2016). Edward D. Goldberg's proposal of "the mussel watch": reflections after 40 years. *Marine pollution bulletin*, *110*(1), 501-510.

Fernández, B., & Albentosa, M. (2019). Insights into the uptake, elimination and accumulation of microplastics in mussel. *Environmental pollution*, *249*, 321329.

Fernández, B., Campillo, J. A., Martínez-Gómez, C., & Benedicto, J. (2012). Assessment of the mechanisms of detoxification of chemical compounds and antioxidant enzymes in the digestive gland of mussels, Mytilus galloprovincialis, from Mediterranean coastal sites. *Chemosphere*, *87*(11), 1235-1245.

Fernández, B., Campillo, J. A., Martínez-Gómez, C., & Benedicto, J. (2011). Micronuclei and other nuclear abnormalities in mussels (Mytilus galloprovincialis) as biomarkers of cyto-genotoxic pollution in mediterranean waters. *Environmental and molecular mutagenesis*, *52*(6), 479-491.

Fossi, M. C., Pedà, C., Compa, M., Tsangaris, C., Alomar, C., Claro, F., ... & Baini, M. (2018). Bioindicators for monitoring marine litter ingestion and its impacts on Mediterranean biodiversity. *Environmental Pollution*, *237*, 1023-1040.

Fulton, M. H., & Key, P. B. (2001). Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environmental Toxicology and Chemistry: An International Journal*, *20*(1), 37-45.

galloprovincialis reared at the vicinities of an aquaculture station. *Chemosphere*, *280*, 130575.

GESAMP, 2016. Sources, fate and effects of microplastics in the marine environment (part 2). Available: http://www.gesamp.org/publications/microplastics-in-themarineenvironment-part-2

Hermabessiere, L., Paul-Pont, I., Cassone, A. L., Himber, C., Receveur, J., Jezequel, R., ... & Soudant, P. (2019). Microplastic contamination and pollutant levels in mussels and cockles collected along the channel coasts. *Environmental Pollution*, *250*, 807-819.

Hudak, P.F. (2004) "Boron and selenium contamination in south Texas groundwater," *Journal of Environmental Science and Health, Part A- Toxic/Hazardous Substances & Environmental Engineering*, 39(11-12), pp. 2827–2834.

Iheanacho, S. C., & Odo, G. E. (2020). Dietary exposure to polyvinyl chloride microparticles induced oxidative stress and hepatic damage in Clarias gariepinus (Burchell, 1822). *Environmental Science and Pollution Research*, *27*(17), 21159-21173.

Kaaya, A., Najimi, S., Ribera, D., Narbonne, J. F., & Moukrim, A. (1999). Characterization of glutathione S-transferases (GST) activities in Perna perna and Mytilus galloprovincialis used as a biomarker of pollution in the Agadir Marine Bay (South of Morocco). *Bulletin of environmental contamination and toxicology*, *62*, 623-629.

Koelmans, A. A., Redondo-Hasselerharm, P. E., Nor, N. H. M., de Ruijter, V. N., Mintenig, S. M., & Kooi, M. (2022). Risk assessment of microplastic particles. *Nature Reviews Materials*, *7*(2), 138-152.

Kolandhasamy, P., Su, L., Li, J., Qu, X., Jabeen, K., & 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.

Kopecka, J., Rybakowas, A., Barˇsiene, J., & Pempkowiak, J. (2004). AChE levels in mussels and fish collected off Lithuania and Poland (southern Baltic). *Oceanologia*, *46*(3).

Lam, P. K., & Gray, J. S. (2003). The use of biomarkers in environmental monitoring programmes. *Marine Pollution Bulletin*, *46*(2), 182-186.

Li, J., Lusher, A. L., Rotchell, J. M., Deudero, S., Turra, A., Bråte, I. L. N., ... & Shi, H. (2019). Using mussel as a global bioindicator of coastal microplastic pollution. *Environmental pollution*, *244*, 522-533.

Li, W. C., Tse, H. F., & Fok, L. (2016). Plastic waste in the marine environment: A review of sources, occurrence and effects. *Science of the total environment*, *566*, 333-349.

Lionetto, M. G., Caricato, R., & Giordano, M. E. (2019). Pollution biomarkers in environmental and human biomonitoring. *The Open Biomarkers Journal*, *9*(1).

Livingstone, D. R., Lips, F., Martinez, P. G., & Pipe, R. K. (1992). Antioxidant enzymes in the digestive gland of the common mussel Mytilus edulis. *Marine biology*, *112*, 265-276.

Lockwood, B. L., Sanders, J. G., & Somero, G. N. (2010). Transcriptomic responses to heat stress in invasive and native blue mussels (genus Mytilus): molecular correlates of invasive success. *Journal of Experimental Biology*, *213*(20), 35483558.

Masiá, P., Ardura, A., & García-Vázquez, E. (2021). Virgin polystyrene microparticles exposure leads to changes in gills DNA and physical condition in the Mediterranean mussel Mytilus galloprovincialis. *Animals*, *11*(8), 2317.

MEDSEALITTER Consortium. (2019). Common Monitoring Protocol for Marine Litter. In *Interegg Med MEDSEALITTER Project*.

Nesto, N., Bertoldo, M., Nasci, C., & Da Ros, L. (2004). Spatial and temporal variation of biomarkers in mussels (Mytilus galloprovincialis) from the Lagoon of Venice, Italy. *Marine environmental research*, *58*(2-5), 287-291.

Ohe, T., Watanabe, T., & Wakabayashi, K. (2004). Mutagens in surface waters: a review. *Mutation Research/Reviews in Mutation Research*, *567*(2-3), 109-149.

Orban, E., Di Lena, G., Nevigato, T., Casini, I., Caproni, R., Santaroni, G., & Giulini, G. (2007). Nutritional and commercial quality of the striped venus clam,

Orbea, A., Ortiz-Zarragoitia, M., Solé, M., Porte, C., & Cajaraville, M. P. (2002). Antioxidant enzymes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve molluscs, crabs and fish from the Urdaibai and Plentzia estuaries (Bay of Biscay). *Aquatic toxicology*, *58*(1-2), 75-98.

Parisi, M. G., Mauro, M., Sarà, G., & Cammarata, M. (2017). Temperature increases, hypoxia, and changes in food availability affect immunological biomarkers in the marine mussel Mytilus galloprovincialis. *Journal of Comparative Physiology B*, *187*, 1117-1126.

Plazzi, F., & Passamonti, M. (2010). Towards a molecular phylogeny of Mollusks:

Revel, M., Lagarde, F., Perrein-Ettajani, H., Bruneau, M., Akcha, F., Sussarellu, R., ... & Mouneyrac, C. (2019). Tissue-specific biomarker responses in the blue mussel Mytilus spp. exposed to a mixture of microplastics at environmentally relevant concentrations. *Frontiers in Environmental Science*, *7*, 33.

Schiedek, D., Broeg, K., Baršienė, J., Lehtonen, K. K., Gercken, J., Pfeifer, S., ... & Schneider, R. (2006). Biomarker responses as indication of contaminant effects in blue mussel (Mytilus edulis) and female eelpout (Zoarces viviparus) from the southwestern Baltic Sea. *Marine Pollution Bulletin*, *53*(8-9), 387-405.

Sigler, M. (2014). The effects of plastic pollution on aquatic wildlife: current situations and future solutions. *Water, Air, & Soil Pollution*, *225*, 1-9.

Stankovic, S., & Jovic, M. (2012). Health risks of heavy metals in the mediterranean mussels as seafood. *Environmental chemistry letters*, *10*, 119-130.

Stankovic, S., Jovic, M., Stankovic, A. R., & Katsikas, L. (2012). Heavy metals in seafood mussels. Risks for human health. *Environmental Chemistry for a Sustainable World: Volume 1: Nanotechnology and Health Risk*, 311-373.

Sun, T., Zhan, J., Li, F., Ji, C., & Wu, H. (2021). Evidence-based meta-analysis of the genotoxicity induced by microplastics in aquatic organisms at environmentally relevant concentrations. *Science of the Total Environment*, *783*, 147076.

Sureda, A., Box, A., Enseñat, M., Alou, E., Tauler, P., Deudero, S., & Pons, A. (2006). Enzymatic antioxidant response of a labrid fish (Coris julis) liver to environmental caulerpenyne. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *144*(2), 191-196.

Tsangaris, C., Kormas, K., Strogyloudi, E., Hatzianestis, I., Neofitou, C., Andral, B., & Galgani, F. (2010). Multiple biomarkers of pollution effects in caged mussels on the Greek coastline. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *151*(3), 369-378.

Tsangaris, C., Panti, C., Compa, M., Pedà, C., Digka, N., Baini, M., ... & Fossi, M. C. (2021). Interlaboratory comparison of microplastic extraction methods from marine biota tissues: A harmonization exercise of the Plastic Busters MPAs project. *Marine Pollution Bulletin*, *164*, 111992.

Tsangaris, C., Vergolyas, M., Fountoulaki, E., & Nizheradze, K. (2011). Oxidative stress and genotoxicity biomarker responses in grey mullet (Mugil cephalus) from a polluted environment in Saronikos Gulf, Greece. *Archives of environmental contamination and toxicology*, *61*, 482-490.

Urban Jr, E. R., & 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.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., & Koehler, A. (2007). The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutantinduced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *146*(3), 281-300.

Webb, S., Gaw, S., Marsden, I. D., & McRae, N. K. (2020). Biomarker responses in New Zealand green-lipped mussels Perna canaliculus exposed to microplastics and triclosan. *Ecotoxicology and Environmental Safety*, *201*, 110871.

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

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

Wu, F., Wang, Y., Leung, J. Y., Huang, W., Zeng, J., Tang, Y., ... & Cao, L. (2020). Accumulation of microplastics in typical commercial aquatic species: a case study at a productive aquaculture site in China. *Science of the Total Environment*, *708*, 135432.

Zbyszewski, M., & Corcoran, P. L. (2011). Distribution and degradation of freshwater plastic particles along the beaches of Lake Huron, Canada. *Water, Air, & Soil Pollution*, *220*, 365-372.

Zhang, F., Man, Y. B., Mo, W. Y., Man, K. Y., & Wong, M. H. (2020). Direct and indirect effects of microplastics on bivalves, with a focus on edible species: A mini review. *Critical reviews in environmental science and technology*, *50*(20), 2109-2143.