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«Επιδράσεις φαρμακευτικών ουσιών και μικροπλαστικών στο μύδι *Mytilus galloprovincialis Lamarck 1819*»

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«Effects of pharmaceuticals compounds and microplastics on mussels *Mytilus galloprovincialis Lamarck 1819*»

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Abstract

Pharmaceutical compounds are widely used in the daily life of humans not only for human use but also for treating household animals. Microplastics are also well used in daily life mainly as a cosmetic ingredient. They are transported in the marine environment via urban sewage. The fate of those compounds is unknown and provokes many questions about their effects on marine organisms.

The present study aimed to examine the responses of a typical marine bioindicator, the filter feeding *Mytilus galloprovincialis*, towards pharmaceutical compounds and microplastics. The mussels were exposed for 29 days to 1,5μg/ L clarithromycin, 0,5 μg/ L venlafaxine, 0,8 mg/ L microplastics and the mixture of the forementioned under laboratory conditions, and they were investigated for different sublethal responses. A set of biomarkers indicative of neurotoxicity (acetycholinesterase), oxidative stress (catalase) and biotransformation (glutathione S transferase) was applied.

Results showed effects of clarithromycin and venlafaxine exposure on catalase activity of mussels and effects of venlafaxine and polysterene microplastics on glutathione S transferase activity, suggesting induction of antioxidant defense and/ or biotransformation. Acetycholinesterase activity did not show any significant differences among mussels exposed to pharmaceuticals or microplastics and control ones.

Overall, our results support the hypothesis that human pharmaceuticals can lead to significant effects on non-target species and that bivalve mollusks may represent sensitive organisms for the action of these compounds in the environment. A more integrated study on the effects of pharmaceutical compounds and microplastics could include the use of more biomarkers and the extent of the exposure period.

Key words: clarithromycin, venlafaxine, polystyrene, microplastics, biomarkers, *M. galloprovincialis*

Table of Contents

1. Introduction

1.1. Pharmaceutical compounds and microplastics as emerging pollutants in the marine environment

Emerging pollutants are chemicals that are not commonly monitored but have the potential to enter the environment and cause adverse ecological and human health effects (Geissen et al. 2015). Their fate, behavior and ecotoxicological effects are not well understood. Emerging pollutants include among other substances, pharmaceuticals and microplastics.

The last decades, a big variety of pharmaceutical compounds are of increasing interest to society, either in agriculture or in humans, regarding growth and robustness (Heberer 2002, Kim et al. 2007). The usage and the consumption of the compounds have been increased because of the creation of new drugs, the population growth, new target age groups, the new uses of existing drugs as well as the generic availability of pharmaceuticals (Daughton 2003, Jelic′ et al. 2012). Pharmaceuticals, such as analgesics, antibiotics, antiseptics, hormones, and antidepressants are given to the patients as in a liquid or a solid form. Those compounds cannot be fully digested and may be excreted partially unmetabolized by human patients (Kummerer 2010). Then, they are rinsed out in the environment through urban sewage (Halling-Sorensen et al. 1998, Song et al. 2008). Investigations indicated that some pharmaceutically active compounds are nearly ubiquitous at low concentrations in water bodies that receive Sewage Treatment Plant (STP) effluent (Stackelberg et al. 2004). Substances are administered either metabolized and excreted or unchanged (Carlsson et al. 2006) in the marine environment. The existence of those substances in the marine environment raises questions and concerns about their occurrence over the last decade (Fent et al. 2006, Boxall et al. 2012) that may lead to a long-term exposition of aquatic organisms (Robinson et al. 2005) and affect their physiology (Hernando et al. 2004, 2006) with unknown consequences to aquatic ecosystems.

Plastics are synthetic organic polymers, which are parts of polymerization of monomer compounds, and derive from oil or natural gas (Derraik 2002, Rios et al. 2007, Thompson et al. 2009). After less than one century of existence, plastic wastes constitute about 60-80% of the total marine litter (Derraik 2002). Plastic waste worldwide is

distributed to surface waters and sediments within the marine environment, reflecting their widespread use. Polymer products have the ability to resist physical and biological decomposition for several years (Galgani et al. 2000, Moore et al. 2001, Katsanevakis et Katsarou 2004, Sudhakar et al. 2007a, 2007b, Andrady et Neal 2009). Marine litter distribution and abundance is controlled by human activities and hydrological factors (e.g. garbage, accidental disposal, ocean circulation) with an excess of 200,000 pieces of plastic found in one square kilometer of water in the North Atlantic subtropical cycle (Galgani et 2000, Katsanevakis & Katsarou 2004, Morishige et al. 2007, Law et al. 2010).

A great scientific interest is currently being directed to microplastics. Microplastics are small plastic particles less than 5mm (Imhof et al. 2013) which are used as raw material in the plastics industry, as scrubs in cosmetics (Derraik 2002, Ryan et al. 2009, Thompson et al. 2004), or derive from macroscopic debris after chemical, physical and biological fragmentation (Barnes et al. 2009).

Microplastics can be ingested accidentlally by many marine invertebrates during their feeding because of their small size range and the nature of ingestion by various organisms such as filter feeders (mussels, barnacles) (Browne et al. 2008).

Both pharmaceuticals and microplastics enter the marine environment via wastewater treatment plants thus can be found simultaneously in receiving waters. Furthermore there is evidence that pharmaceuticals can be sorbed on microplastics (Wu et al. 2016) which could act as carriers of these chemicals to marine organisms.

1.2. Biomarkers of marine pollution

The term biomarker is a compound word formed by joining “bio”, referring to the biological response and “marker” which is serving as a guide (Schlenk 1999). With the definition of biomarker we refer to ‘‘any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status”. Thus biochemical, physiological, histological, morphological and behavioral measurements that signal exposure to and/or adverse effects of environmental chemicals are to be considered as biomarkers (Walker et al. 2006, Depledge 1994).

Biomarkers are well-used by many groups of scientists as measures of chemical contamination in order to determine pollution effects. Biomarkers are used to determine whether or not organisms are physiologically ‘normal’. It is notable to know at what point a departure from the normal, healthy state (homeostasis) is likely to affect the attitude of the organism (survival, growth or reproduction). With regard to this fact, biomarkers may be used as early warning signals for effects on the population level (Depledge et Fossi 1994, Lagadic et al. 1994, Den Besten 2010).

The advantage of the usage of biomarkers (Depledge et al 2009) compared to conventional tissue residue analyses, is that they indicate when a pollutant has induced a compensatory response within the organism (indicating that the chemical is not in an inert form) whereas tissue chemical analysis cannot. Moreover, biomarker responses can reveal the presence of contaminants that were not suspicious initially. Thirdly, biomarker responses usually remain long after a temporary exposure to a contaminant that has subsequently been degraded and is no longer detectable. Finally, biomarker analyses are much easier to perform and are considerably less expensive than a wide range of chemical analyses.

Biomarkers can be divided into three categories: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. The main role of exposure biomarkers is to measure the substance or its metabolic pathway in an organism. The main aim for using biomarkers of exposure in biological monitoring is the prevention of adverse effects on the monitored ecosystem. A biomarker of exposure is not a direct indicator, but it may be a predictor of a potential health effect. The role of biomarkers of exposure is that they can detect exposure and absorbed dose in organisms before the onset of observed health effects. (Lowry 1995).

Biomarkers of effect signal an adverse effect on the organism. There is a difficulty in defining “adverse effects” or “stress”. Stress was categorically divided into direct stress (resulting from stressor exposure) and indirect stress (resulting from disruption in energy availability, storage or utilization in repair or development) (Adams 1990). The foundational concept of the biomarker approach is to assess adverse effects or stress which is based on the hypothesis that the effects of stress are typically manifested at different levels of biological organization before disturbances are realized at the

population community or ecosystem levels (Adams 1990). That type of initial effects is present at the molecular level primarily with the induction of cellular defense systems.

The last category is the biomarkers of susceptibility. The overall aim of biomarker studies is to predict the ecosystem effects based on molecular or whole animal endpoints, and after that the areas of susceptibility may occur throughout the time. It is very important to identify susceptible individuals. This is a significant component of most risk assessment paradigms, since various uncertainty factors are often included to provide safe assessments for individuals that may be more sensitive than tested organisms (Calabrese & Baldwin 1996). Therefore, biomarkers of susceptibility can be defined as indicators of the mechanistic processes that provokes diversity among the compartment in the continuum between exposure and effect (Barrett et al. 1997).

Measurements of sets of biomarkers are proposed for an integrated assessment of pollution effects. Researchers aim to define the necessary biomarkers which will reflect the present and future problems of marine ecosystem. Several enzymes have been used in research programs as biomarkers for tracking pollution in the Mediterranean sea (Solé, et al. 1994, Burgeot et al. 1996). For example, acetycholinesterase inhibition has been successfully applied in several studies for pollution assessment in the Mediterranean sea (Tsangkaris et al. 2010).

1.3. Acetylcholinesterase

Acetylcholinesterase (AChE) is an enzyme and it is part of cholinergic brain synapses and neuromuscular junctions. Its main role is to intercept the transmission via a rapid hydrolysis of the cationic neurotransmitter acetylcholine. This action of the biological pathway is necessary in order to understand the effect of acetylcholinesterase inhibitors in treatments (Tõugu 2001). Acetylocholinesterase has been widely used as a biomarker of neurotoxicity.

It has been demonstrated, in a field as well as in laboratory studies, that AChE is inhibited by organophosphorus compounds (OPs) and carbamates, substances widely used in agriculture as pesticides. As a consequence, AChE has been employed as a useful biomarker of exposure to pesticides in biomonitoring studies (Escartín et Porte 1997, Radenac et al. 1998, Mora et al. 1999, Dailianis et al. 2003). However,

acetylocholinesterase is also inhibited by several other pollutants such as metals and surfactants (Tsangkaris et al. 2010). As many other biomarkers, AChE shows seasonal variations. For example, in *M. galloprovincialis* at Ebro Delta (Spain), the highest AChE activity was detected in winter, and the lowest in summer (Escartín et Porte 1997).

1.4. Catalase

Catalase (CAT) is an enzyme which is found almost in all organisms which are living in the air. It disintegrates the hydrogen peroxide to water and oxygen (Chelikani et al. 2004). It composes one of the very important enzymes in reproductive reactions. Catalase is very active and one molecule could transform millions of molecules of hydrogen peroxide to water and oxygen each second (Goodsell 2004).

The major role of catalase is the destruction of hydrogen peroxide by the peroxidatic or "catalatic" reactions. This is explained both by the intracellular location of the enzyme, the similarity of its cellular distribution to that of the cytochromes, and the protective effect of catalase for cells exposed to ionizing radiation. This fore mentioned effect is presumed to be owing to the destruction of the hydrogen peroxide generated by the ionizing radiation. Catalase seems to act in parallel with other systems like the glutatthione peroxidase and the methemoglobin reductases, that circumvent the accumulation of methemoglobin, either by preventing the oxidation of hemoglobin by peroxide or by reducing the methemoglobin to hemoglobin as fast as it is formed (Deisseroth & Dounce 1970).

There is particular interest in antioxidant enzymes that oppose ROS-induced oxidative damage. Catalase was one of the first enzymes proposed to be an effective marker of oxidative stress (Livingstone et al. 1993). Furthermore, catalase has a variety of applications as biomarker. It can not only give precise results from the response of the organisms but it can also be involved in degradation of contaminant (Jiang et al. 2017).

1.5. Glutathione-S-Transferase

Glutathione-S-Transferase (GST) is a key enzyme of phase II of the biotransformation of organic pollutants and some endogenous compounds. It is a tripeptide thiol found in living organisms. It occurs in animals' cells as well as in most

plants and bacteria. The enzyme catalyzes the conjugation of the tripeptide glutathione with electrophilic substrates, including, among xenobiotics, above all polycyclic aromatic hydrocarbons and polychlorinated biphenyls, and among natural metabolites, steroid sex hormones, prostaglandins, fatty acids, and their derivatives (Kolesnichenko & Kulinskii 1989).

GST belongs to biomarkers of the defensive response that develops in an organism under the influence of various pollutants, in contrast to, for instance, lipid peroxidation or ruptures in DNA chains, which are biomarkers of damage (De Lafontaine et al. 2000).

The glutathione S-transferase (GST) enzyme exhibits many of the required characteristics of biomarkers of organ damage as, i.e. specific localisation, high cytosolic concentration and relatively short half-life. The role of GST as early indicators of organ damage is applicable to both human and animal models. GST permits the identification of specific areas of damage within a particular organ (Kilty et al. 1998). It can take part in detoxification and provide antioxidant defense to xenobiotically induced oxidative stress are the major contributors to xenobiotics detoxification pathways in fish. (Yu et al 2008).

1.6. Biology of the mussel *Mytilus galloprovincialis*

The sessile mussel *Mytilus galloprovincialis Lamarck 1819* is found on subtidal shores and it is a very common in the north hemisphere. It is considered to be endemic to the Mediterranean (Wonham 2004, Carlton 1999) and it ranges from Mexico to central California. In the Northeast Pacific, *M. galloprovincialis* its presence is confirmed throughout the inner waters of Washington (Suchanek et al. 1997, Anderson et al. 2002*).*

M. galloprovincialis follows distinctive distributional patterns in estuarine and coastal environments. It is a filter feeder and it absorbs its food and releases waste materials to the overlaying waters. Its habitat supplies growing phytoplankton and an environment for nutrient recycling (Dame 1993).

Mussel is a gonochoristic species which displays two spawning periods, in April and late summer (July-August). Individuals in spawning conditions can be identified throughout the year. The sex ratio fluctuates about 1:1 and only a small amount (2%) has

been estimated as hermaphrodites (Sedik et al. 2010).At the Table 1.1 the taxonomic classification is presented.

Table 1.1 Taxonomic Classification of *Mytilus galloprovincialis* (*Lamarck 1819) (www.marinespecies.org).*

Phylum	Mollusca
Class	Bivalvia
Order	Mytilida
Family	Mytilidae
Genus	*Mytilus*
Species	*Mytilus galloprovincialis*

The sessile bivalve *Mytilus galloprovincialis* is composed of two halves. These two are joined by a ligament and from interior are kept by a muscle. The movement is achieved by swelling of foot with pumping of blood in it. Mussels are breathing in accordance to the operation of gas exchange using their gills and the mantle. The food is transported to the organisms by siphons of input and output at gills and mouth. After that, food is transmitted to a cylindric stomach. A mussel's byssus is produced from within the shell by a byssal gland with which the mussels are attached to the substrate (Στάικου κα. 2015).

1.7. *M. galloprovincialis* as sentinel species for pollution assessment

The mussel *Mytilus galloprovincialis* is one of the most common bivalves, used in environmental studies as bioindicator. It is widely used as sentinel species for monitoring contamination in coastal ecosystems (Box et al. 2007, Santovito et al. 2005, Saavedra et al. 2004).

The mussel is sessile and feeds by filtering the seawater thus accumulating contaminants at high concentrations. It can bio-accumulate at concentration levels found in seawater (Viarengo & Canesi 1991). Mussels have low decontamination kinetics (Wang et al. 1996) and are resilient but they prone to the accumulation of pollutants and

change their physiological functions (Leinio & Lehtonen 2005, Gorbi et al. 2008, Tsangaris et al. 2010).

Because of its sedentary nature, the mussel follows the changes in marine habitat and it can give precise results in different time about the changes of the environment (Connell 1970). Many researchers have investigated the accumulation of contaminants and biomarkers in mussels (Canesi et al. 2007a, Gonzalez-Rey et al. 2014).

1.8. Aim

The present work aims at studying the effects of two pharmaceutical compounds, clarithromycin and venlafaxine, as well as polysterene microplastics on *Mytilus galloprovincialis* in laboratory conditions using a set of biomarkers indicative of neurotoxicity (AChE), oxidative stress (CAT) and biotransformation (GST). Results are likely to contribute to understanding the environmental effects of pharmaceutical compounds in combination with microplastics in the aquatic ecosystem.

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2. Methodology

2.1. Chemicals and reagents

Clarithromycin (CAS NUMBER 81103-11-9) and Venlafaxine (CAS NUMBER 99300-78-4), were purchased from the company Sigma Aldrich.

Polystyrene (PS) in powder form was provided by a local plastic supplier. PS microplastic particles were sieved at 63 μm and the < 63 μm particles were used.

The Bradford reagent was purchased from Bio Rad and all other chemicals were purchased from Sigma Aldrich, BIO-RAD and Merck. Mussel feed was Phytoplex from Kent Marine. Tropic Marin® Sea Salt was used to prepare artificial sea water.

2.2. Choice of test substances

Two pharmaceuticals (clarithromycin and venlafaxine) and polystyrene microplastic particles were chosen as they are all present in waste water treatment plant (WWTP) effluents and thus can be found together in the receiving waters.

Clarithromycin is a compound used as human and veterinary antibiotic which is derived from erythromycin. It is especially used for treating human pathogen infections like pharyngitis/tonsillitis due to *Streptococcus pyogenes*, pneumoniae; community acquired pneumonia or bronchitis due to *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, or *Chlamydia neumoniae*; uncomplicated skin infections due to *Staphylococcus aureus*, or *Streptococcus pyogenes*; and disseminated mycobacterial infections due to *Mycobacterium avium*, or *Mycobacterium intracellulare*. (Womble et al. 2006). It is widely used for its effectiveness in the treatment of *Rhodococcus equi* infection in foals (Jacks et al. 2003) and it is also prescribed for cats and dogs.

Clarithromycin has been introduced to the environment through urban waste waters. It constitutes risk for terrestrial and aquatic organisms on account of unknown effects in ecosystem (De la Torre et al. 2012). Clarithromycin is proposed to be included in the first watch list that is developed under the Environmental Quality Standards Directive (2008/105/EC) as a compound with doubtful consequences (Carvalho et al. 2015). Clarithomycin has been detected in Saronikos Gulf waters (Alygizakis et al. 2016).

Venlafaxine is an antidepressant which can be designed from (R/S)-1-[2-(dimethylamino)-1(4-methoxyphenyl)ethyl] cyclohexanol hydrochloride or (±)-1-[α-[(dimethylamino)methyl]-pmethoxybenzyl] cyclohexanol hydrochloride and its most common trade name is Effexor.

It is believed that it is associated with the neurotransmitters' activity in the central nervous system (Effexor XR). It intercepts the synapses between the noradrenaline and serotonin (Redrobe et al. 1998). It is used for major depressive disorder and certain other psychiatric disorders.

Recent researches have detected antidepressant drugs in seawater of the Saronikos Gulf using the largest in terms of number of evaluated analytes and provided the first evidence of the presence of venlafaxine in seawater (Alygizakis et al. 2016). The detection of venlafaxine in Saronikos Gulf waters may be related to increased use in the last years owing to the financial crisis which our country is facing.

Microplastics are small plastics granules or fragments less than 5mm (Imhof et al. 2013) which are used in scrubs and other cosmetics products, as raw materials by the plastic industry or derive from the segmentation of bigger plastics (Derraik 2002, Ryan et al. 2009, Thompson et al. 2004).

Polystyrene (PS), also known by its most common brand name Styrofoam, is a petroleum-based, non-renewable plastic made from the styrene monomer. It is widely used in packaging materials, daily products such as CDs cases and cups. It is commonly found as marine litter on coastlines and in the oceans. The characteristics of polystyrene have as a result of the rapid fragmentation the endurance for a long time in the environment. That's why, some efforts are done to reduce the unsustainable use of polystyrene in everyday products by encouraging collection and recycling in order to prevent from environmental pollution (Institute for European Environmental Policy, IEEP). PS particles were used as they are among the most commonly found microplastics in the marine environment (Cole et al. 2011). Due to their neutral buoyancy in seawater, PS particles can remain suspended in the water column and thus are available to filter feeding organisms (Karami et al. 2017).

2.3. Stock solutions of the test substances

A stock solution of clarythomycin (100 mg/L) was prepared in methanol. For this, 0,01 gr of clarithromycin was weighed and placed into a 100 ml volumetric flask that was filled with methanol. A specific volume of the stock solution (150 μl) was added in eppendorf tubes that remained in N_2 air until evaporation. After that, the eppendorf tubes were filled with 2 ml deionized water and mixed in a vortex. This solution was added to seawater in the exposure tanks. The clarythomycin concentration which was given to the mussels in the exposure tanks was 1,5 μg/L. The original stock solution was maintained in the freezer at -20 °C.

The stock solution of venlafaxine (10 mg/L) was prepared in deionised water. The solution was prepared by weighing 0,0564 gr and adding in a 500 ml volumetric flask. The rest was filled with deionized water. A concentration of 0,5 μg/L venlafaxine was provided to the mussels by adding the stock solution to seawater in the exposure tanks. The stock solution of venlafaxine was kept in the refrigerator at 4 °C

A stock solution PS particles (10 g /L) was prepared in artificial seawater (prepared with deionized water). An amount of 0,5 gr of PS microplastics were weighed and homogenized with artificial seawater up to 50 ml. This solution was added to seawater in the exposure tanks to achieve a concentration of 0,8 mg/L.

2.4. Mussels exposure

The mussels were purchased from the mussel culture «Sevastis» which is situated in Saronikos Gulf at the region of Nea Peramos.

A preliminary experiment took place to test the experiments' main conditions. Individuals of similar size ranging from 6 to 6,9 cm were chosen. Overall, 75 mussels were collected, cleaned of epibiotes and the byssus threads (figure 2.1), and separated randomly in 3 tanks containing 5L of artificial seawater (Sera ® Marin Salt). In each tank 25 individuals were hosted.

Figure 2.1 Mussels before and after cleaning.

Salinity was 38 ‰ and the temperature was 18 ± 1 ^{o}C. The individuals were acclimatized for one week in aerated conditions.

Then the mussels were exposed to venlaflaxine (1,5 μg/L) and clarithomycin (1,5 μg/L) for 13 days. Sea water was changed every second day, redosing the above concentrations with stock solutions.

The mussels were fed daily on *Dunaliella sp*. culture and the amount of food for each mussel was 8 * 10^{6} cells/ mussel. Every day, the tanks were checked for dead mussels which were removed and recorded.

For the main experiment, the size of mussels ranged from 5 to 6,5 cm. In whole, 375 mussels were collected, cleaned of epibiotes and the byssus threads (figure 2.1), and separated randomly in 15 tanks containing 5 L of artificial seawater (Tropic Marin® Sea Salt). In each tank 25 individuals were hosted.

Figure 2.2 Mussels exposure to clarithomycin, venlaflaxine and PS.

Salinity, temperature and aeration remained the same as during the preliminary experiment.

After one week acclimation, the mussels were exposed to venlaflaxine (0,5 μg/L), clarithomycin (1,5 μg/L), PS (0,8 mg/L) and their mixture (venlaflaxine 0,5 μg/L, clarithomycin 1,5 μg/L, PS 0,8 mg/L) for 29 days (figure 2.2).

The chosen exposure concentration is higher than that typically found in the sea (from a few units to hundreds of ng/L, Alygizakis et al. 2016, Lolic et al. 2015, Gros et al. 2012), but still environmentally realistic for particularly challenged sites (up to tens of mg/L, Togola & Budzinski 2008). All treatments were in triplicate. Three tanks with no added substances served as controls. Sea water was changed every second day, redosing concentrations solutions.

The mussels were fed daily on phytoplankton Phytoplex Kent and the amount of food for each mussel was $8 * 10^6$ cells/ mussel. Every day, the tanks were checked for dead mussels which were removed and recorded.

2.5. Mussels sampling

In two periodical intervals (15 and 29 days), 12 mussels were sampled from the tanks. In each sampling total length was recorded for each individual. Then, the digestive gland and gills were collected from each mussel.

Figure 2.3 Mussel before extraction of gills and digestive gland.

Three mussels constituted one sample. The tissues were dissected out and were put on cryovials. The whole procedure took place in ice conditions. The cryovials were transported into liquid nitrogen for a couple of minutes with the aim of direct freezing for enzyme activity preservation. Then, the samples were kept in the deep freezer at -80°C.

For the pre experiment, 3 mussels were sampled per tank at 13 days of exposure and one mussel was used per sample. Otherwise, sampling was as described above.

2.6. Acetylcholinesterase activity assay

Acetylcholinesterase hydrolyses acetylothiocholine into thiocholine. In order to determine the acetylcholinesterase activity, the Ellman's method (Ellman et al. 1961) was applied. This method is based on the increase of absorption of yellow color of the Ellman's reagent (DTNB) produced from thiocholine when it reacts with dithiobisnitrobenzoate ion. The absorption is measured at 412 nm.

$$acetylthiocholin \xrightarrow{AChE} thiocholine + acetate$$

$$thiocholine + dithiobidnitrobenzoate \rightarrow yellow\ colour$$

To determine the enzyme activity, gill tissue was used because this tissue shows high AChE activity. The procedure applied was according to Bocquene 2001. Gill samples were cut into pieces and weighed. The samples were homogenized (1:2 w:v) in 0,1M Tris-HCl buffer containing 0,1% Triton x 100, pH 7 with a Potter - Elvehjem homogenizer. The homogenized samples were centrifuged at 10.000 g for 20 minutes.

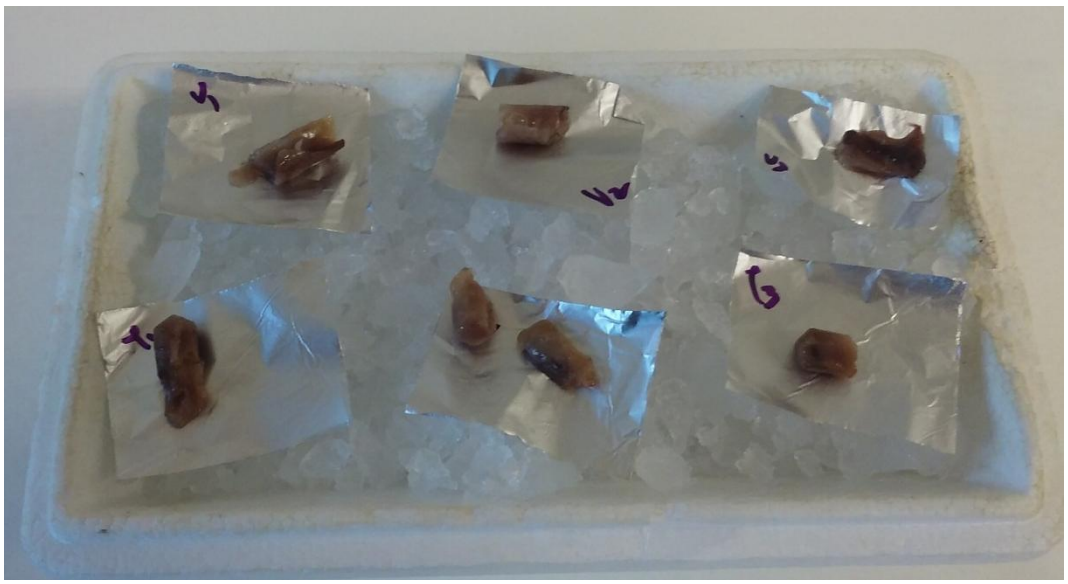

Figure 2.4 Gills remained in ice before the homogenization.

The supernatant was transferred into two eppendorfs and maintained at -80 °C. One supernatant sample was used for AChE activity measurement and the other one for analysis of proteins. The whole procedure took place at 4°C.

Enzymatic activity was determined in a microplate reader (BIOTEK Synergy HTX Multi-Mode Microplate Reader) at 25°C. In each microplate well 60 μL of sample, 290 μl 0,1M Tris-HCl buffer containing 0,1% Triton x 100, pH 7, and 20 μl 0,01 M DTNB were added. The reaction started with the addition of the substrate (10 μl of 0,1 M acetylothiocholine). Absorbance was read at 412 nm every 20 seconds for 2 minutes. Samples were measured in triplicate.

Enzyme activity of acetylcholinesterase was expressed as U/ minute/ mg protein or as nanomoles of acetylothiocholine hydrolysed/ minute/ mg protein. The one unit (U) of acetylcholinesterase activity is the variation of 0,001 of absorbance and 1 Δ of absorbance/ minute/ mg protein corresponds to the hydrolysis of 75 nanomoles of acetylothiocholine.

2.7. Catalase assay

Catalase disintegrates H_2O_2 according to the reaction

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} O_2 + H_2O$$

In order to determine catalase activity, digestive gland tissue was used. The procedure was according to Cohen et al. 1996. Digestive gland samples were cut into pieces and weighed. The samples were homogenized (1:4 w:v) in 100 mM KH_2PO_4/ K_2HPO_4, pH 7,4 with a Potter - Elvehjem homogenizer. The homogenised samples were centrifuged at 10.000 g for 30 minutes. The supernatant was transferred into three eppendorfs and maintained at -80 °C. Two supernatant samples were used for catalase and glutathione-S-transferase activity measurements and the other one for analysis of proteins. The whole procedure took place at 4°C.

Enzymic activity was estimated by the decrease in the concentration of H_2O_2 after 4 min of incubation with the sample. The decrease in H_2O_2 concentration was measured colorimetrically with ferrous ions and thiocyanate on a microplate reader.

$$H_2O_2 + \text{sample} \rightarrow \text{decrease in concentration of } H_2O_2$$

All reagents were kept in an ice bath except for the sulphuric acid which was stored at room temperature. The procedure was as follows.

In tubes of 20 ml were put 4 ml of 0,6 N H_2SO_4 and 1 ml of 10 M $FeSO_4$ were put and mixed with a vortex. For each sample, 4 tubes were prepared in duplicate, for measurements at 1 and 4 minutes period.

In 5 ml tubes were added 50 μl of sample and 850 μl of 10 mM KH_2PO_4/ K_2HPO_4, buffer pH 7 in duplicate in an ice water bath and then mixed by vortex. The reaction was initiated adding 100 μl of 60 mM hydrogen peroxide (H_2O_2) and applying low speed vortex.

At 1 and 4 minutes, 100 µl aliquots were removed, added to the tubes containing the mixture of H_2SO_4 and $FeSO_4$ reagents and stirred. After that, 400 µl of 2,5 M KSCN were added to the tubes and mixed by low speed vortex. The absorption was measured at 490 nm. The procedure was carried out in duplicate for each sample. Aluminum foil was used to cover the samples with a view maintaining the colour stable up to the measurement of absorption.

The amount of catalase was estimated by the H_2O_2 loss and expressed as enzyme units,

$$U/_{protein} = \left[\ln {(A1/A4)}/_{(t_1 - t_4)}\right] * dil/\ protein$$

Where:

A_1 and A_4: Absorption at 1 and 4 minutes respectively and protein is the protein concentration.

2.8. Glutathione S Transferase (GST) assay

The activity of Glutathione S Transferase (GST) was estimated by measuring photometrically the rate of conjugated substrate 1-Chloro-2,4-dinitrobenzene (CDNB) with glutathione (GSH) at 340 nm. The procedure was based on Habig and Jakoby's method (1981). The reaction is the following:

$$CDNB + GSH \xrightarrow{GST} GSH\ CDNB - GS + HCl$$

The preparation of samples for estimation of GST activity was the same as the one followed for catalase.

Enzymatic activity was estimated in a microplate reader at 25°C. In order to prepare the reaction solution, 20 ml of 0.2 M phosphate buffer pH 6,5. In a flask of 30 ml, 0,5 ml CDNB 42 mM and 0,5 ml GSH 1mM were added into a 30 ml flask. In the wells of a microplate, 15 ml of sample were put in triplicate. The reaction was started by adding 200 µl of the reaction mixture in the wells using a multichannel pipette.

The absorbance was measured at 340 nm for 4 minutes at 25°C with intervals of 30 seconds. The enzyme activity of GST was expressed as nanomoles of CDNB conjugate formed /minute/mg protein.

2.9. Measurement of the total concentration of proteins.

The total protein was estimated by the absorbance of blue colour according to Bradford (1976). The absorbance owns to Bradford's reagent at 595 nm. As a norm, BSA (Bovine Serum Albumin) protein was used.

Digestive gland and gill samples were diluted 1/400 and 1/200 respectively. Protein concentration was determined in a microplate reader at 25^{O}C. In order to create the standard curve, a stock solution of BSA was added in the microplate wells with combination of deionized water with a view to retaining a stable volume of 100 μl (0 μl BSA + 100 μl deionized water, 10 μl BSA + 90 μl deionized water … 40 μl BSA + 60 μl deionized water). In other microplate wells, 100 μl of the diluted samples were added. In all wells 280 μl Bradford reagent was added. The absorbance at 595 nm was read after 2 min. Samples were measured in duplicate.

2.10. Data analysis

Biomarker data were analyzed using the SPSS statistical package. In order to check for normality and homogeneity of variance, the Kolmogorov – Smirnov and Levene's tests were applied. Significant differences between each treatment group and control mussels were determined using one-way ANOVA followed by the multiple comparison LSD's test employed as post hoc test. In any case, statistical differences were accepted when $p < 0,05$.

3. Results

3.1. Preliminary experiment

In the pre experiment, the mussels were exposed to venlaflaxine (1,5 µg/L) and clarithomycin (1,5 µg/L) for 13 days. The mortality was high in the venlafaxine exposed mussels. In particular, 9 mussels out of 25 were cleared out during the course of the pre experiment. In clarithromycin and control 3 and 2 mussels were removed respectively. The enzymatic activity didn't determine in clarithromycin exposed mussels because of enzymatic cell lysis.

3.1.1. Acetylcholinesterase activity

No significant difference was observed in acetylcholinesterase (AChE) activity between control and venlafaxine exposed mussels.

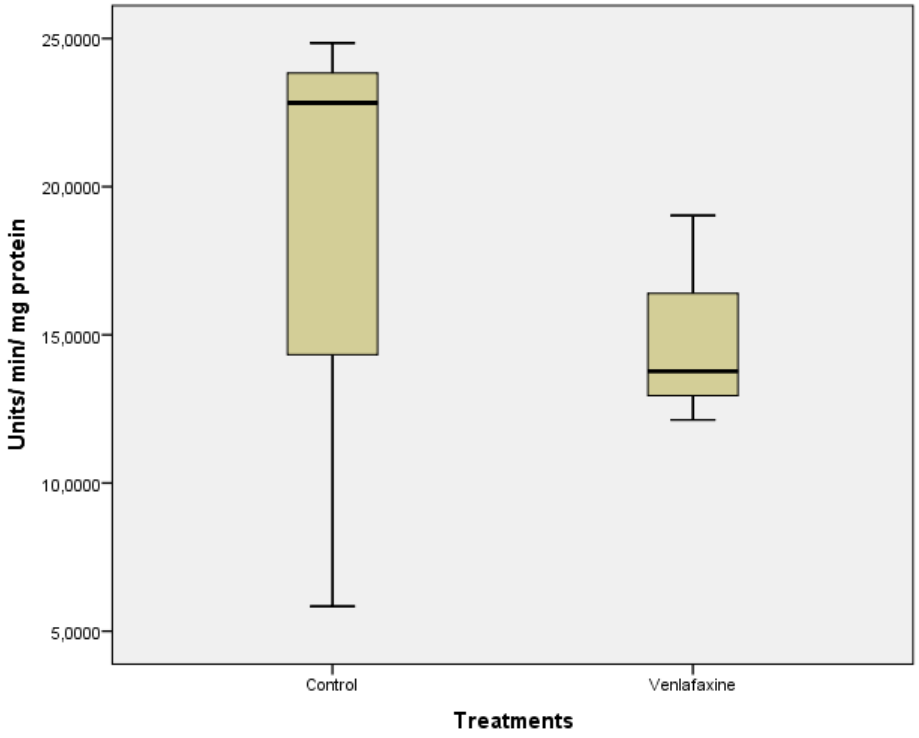

Figure 3.1 Boxplot analysis for acetylcholinesterase (AChE) for the pre experiment. Values are the medians ± standard deviations (n= 3 per treatment). Y axis represent AChE Unitis/ min/ mg proteins.

3.1.2. Catalase activity

No significant difference was perceived in catalase activity between control and venlafaxine exposed mussel.

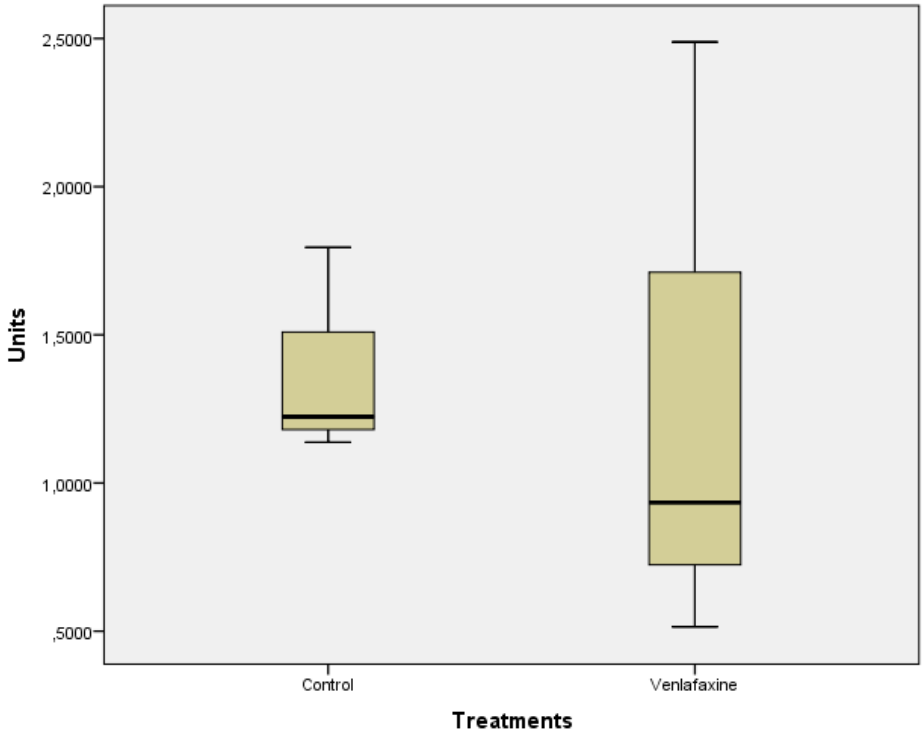

Figure 3.2 Boxplot analysis for catalase for the pre experiment. Values are the medians ± standard deviations (n= 3 per treatment). Y axis represent catalase units.

3.1.2. Glutathione S transferase activity

As it is concerned GST activity, no significant difference was noticed in between control and venlafaxine exposed mussels.

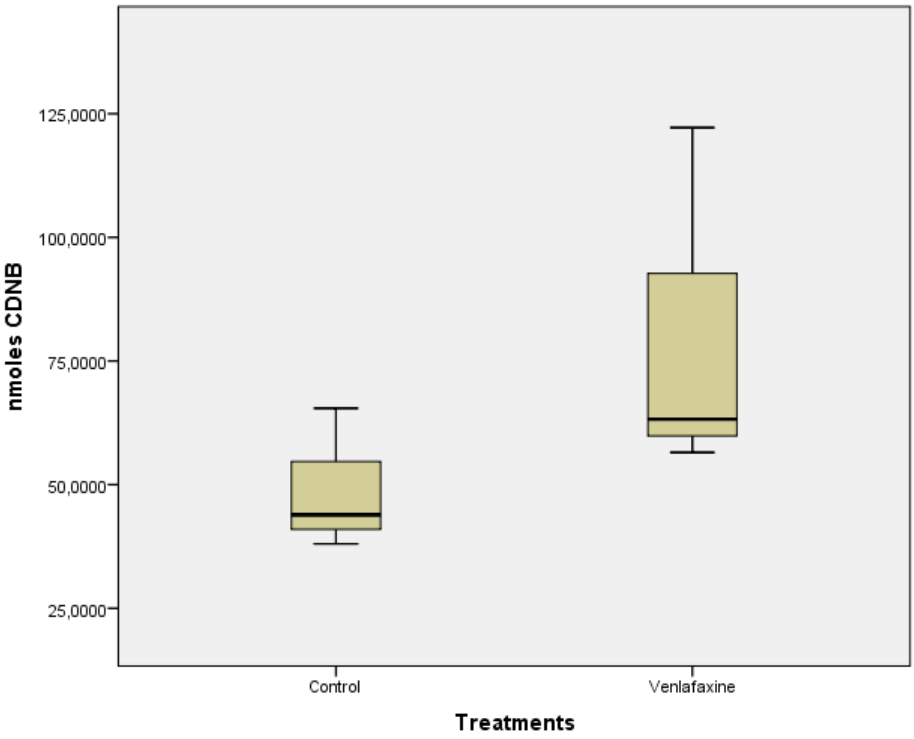

Figure 3.3 Boxplot analysis for glutathione S transferase for the pre experiment. Values are the medians ± standard deviations (n= 3 per treatment). Y axis represent moles of 1-Chloro-2,4-dinitrobenzene (CDNB).

3.2. Main experiment

In the main experiment, the mussels were exposed to venlaflaxine (0,5 μg/L), clarithomycin (1,5 μg/L), PS (0,8 mg/L) and their mixture (venlaflaxine 0,5 μg/L, clarithomycin 1,5 μg/L, PS 0,8 mg/L) for 29 days. No mortality was observed in control tanks. Overall, 19 individuals were cleared out during the experiment. From the clarithromycin treatment 7 mussels were removed, from the venlafaxine treatment 9, from the microplastics treatment 1 and from the mixture treatment 2.

3.2.1. Acetylcholinesterase activity

The effect of the four treatments (clarithromycin 1,5μg/L, venlafaxine 0,5 μg/L, microplastics 0,8 mg/L and mixture) on acetylcholinesterase activity in mussels' gills at two and four weeks of exposure are shown in Figures 3.4 and 3.5. In the control tanks mean AChE activity (Units/ min/ mg protein) was 34,73 ± 7,52 at 15 days and 26,27 ±

10,16 at 29 days, in the clarithromycin tanks was 32,44 ± 16,17 at 15 days and 20,22 ± 9,11 at 29 days, the venlafaxine tanks 49,75 ± 26,55 at 15 days and 22,60 ± 9,13 at 29 days the microplastic tanks 23,24 ± 7,68 at 15 days and 22,13 ± 5,16 at 29 days and the mixture ones 34,00 ± 7,24 at 15 days and 32,66 ± 10,79 at 29 days.

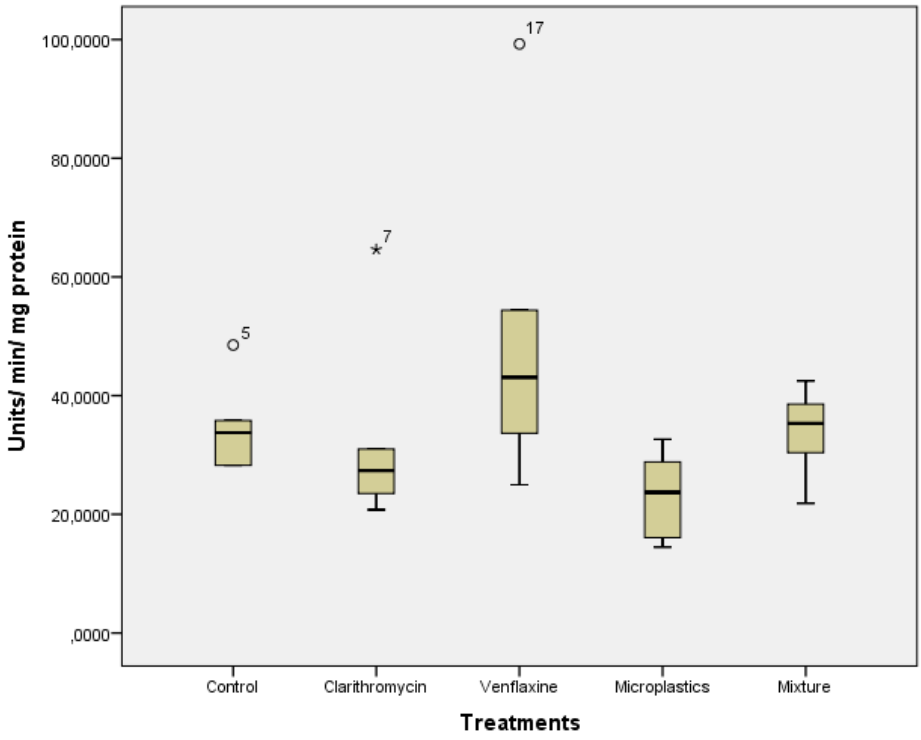

Figure 3.4 Boxplot analysis for acetylcholinesterase for two weeks period. Values are the medians ± standard deviations (n= 6 per treatment). The (*) and the (^{O}) depict the outliers.

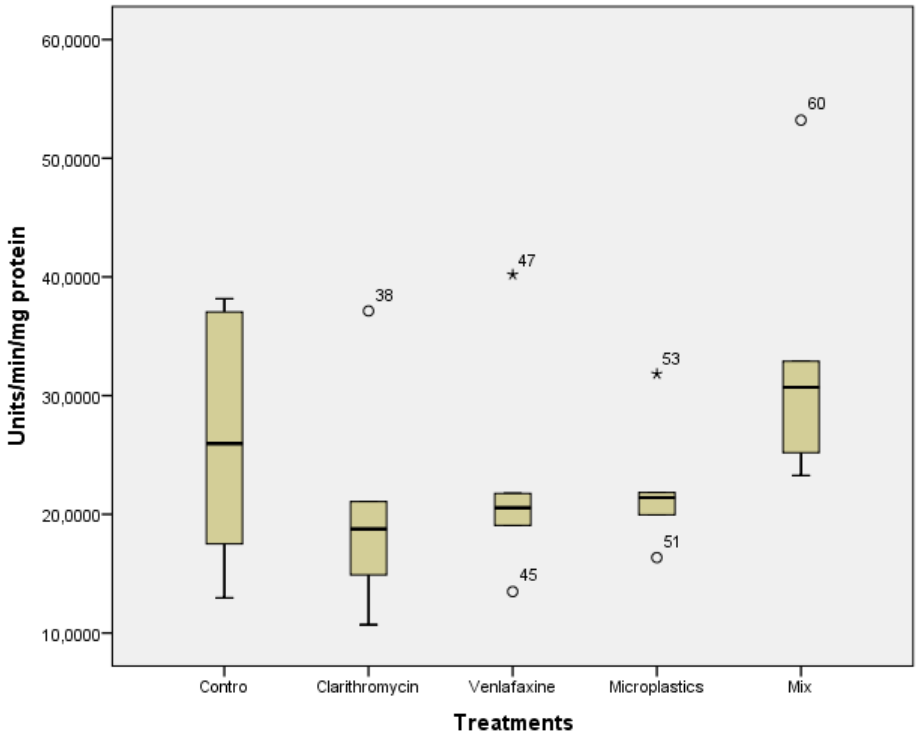

Figure 3.5 Boxplot analysis for acetylcholinesterase for four weeks period. Values are the medians ± standard deviations (n= 6 per treatment). The (*) and the (^{O}) depict the outliers).

Statistical analysis

The Kolmogorov - Smirnov test was performed to check if data followed normal distribution. The test showed that data originated from a normal population as shown in the Table 3.1.

Table 3.1 Kolmogorov-Smirnov's test of normality.

	Kolmogorov-Smirnov[a]		
	Statistic	df	Sig.
Log10_1+AChE activity (Units/min/mg protein)	,071	60	,200*

*This is a lower bound of the true significance.

[a] Lilliefors significance correction

The Levene's test was applied to check for equality of error variances for acetylcholinesterase data. The test showed homogeneity of variance as shown in Table 3.2.

Table 3.2 Levene's test of equality of error variances for acetylcholinesterase data.

F	df1	df2	Sig.
,852	9	50	,573

Since data complied with the assumptions of normality and homogeneity of variance, a univariate analysis was performed. The two way ANOVA showed significant differences in AChE activities among treatments (F= 2,847, P= 0,033) and between sampling periods (F= 12,965, P= 0,001). The interaction of the fore-mentioned factors didn't show any significance (F= 2,224, P= 0,08).

Table 3.3 Two way ANOVA for acetylcholinesterase with treatment and sampling periods as factors.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,710[a]	9	,079	3,695	,001
Intercept	126,569	1	126,569	5926,271	,000
Treatment	,243	4	,061	2,847	,033
Weeks	,277	1	,277	12,965	,001
Treatment * Weeks	,190	4	,048	2,224	,080

a. R Squared = ,399 (Adjusted R Squared = ,291)

Thus a multiple comparison test was performed (LSD) that showed significant differences between three pairs of treatments. Significant differences in AChE activity of mussels were found between the clarithromycin and the mixture treatment (p=0,036), the venlafaxine and the microplastics (p=0,018) and finally the microplastics and mixture

treatments (p=0,009). Ache activities of mussels were lower in the clarithromycin than in the mixture treatment, in the venlafaxine than the microplastics treatment, and in microplstics than the mixture treatment. However, there was no difference between control tanks and treatments.

Concerning sampling period, all the treatments showed decreased activities at four weeks with the highest reduction in the venlafaxine treatment.

Table 3.4 Multiple comparisons for acetylcholinesterase (LSD).

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Clarithr	,081895	,0596618	,176	-,037939	,201730
	Venlafax	-,029879	,0596618	,619	-,149713	,089956
	Micropl	,115967	,0596618	,058	-,003868	,235801
	Mixture	-,046587	,0596618	,439	-,166422	,073247
Clarithromycin	Control	-,081895	,0596618	,176	-,201730	,037939
	Venlaf	-,111774	,0596618	,067	-,231608	,008060
	Micropl	,034071	,0596618	,571	-,085763	,153905
	Mixture	-,128483*	,0596618	,036	-,248317	-,008648
Venlafaxine	Control	,029879	,0596618	,619	-,089956	,149713
	Clarithr	,111774	,0596618	,067	-,008060	,231608
	Micropl	,145845*	,0596618	,018	,026011	,265680
	Mixture	-,016709	,0596618	,781	-,136543	,103126
Microplastics	Control	-,115967	,0596618	,058	-,235801	,003868
	Clarithr	-,034071	,0596618	,571	-,153905	,085763
	Venlaf	-,145845*	,0596618	,018	-,265680	-,026011
	Mixture	-,162554*	,0596618	,009	-,282388	-,042720
Mixture	Control	,046587	,0596618	,439	-,073247	,166422
	Clarithr	,128483*	,0596618	,036	,008648	,248317
	Venlafax	,016709	,0596618	,781	-,103126	,136543
	Micropl	,162554*	,0596618	,009	,042720	,282388

3.2.2. Catalase activity

The effect of the four treatments (clarithromycin 1,5µg/L, venlafaxine 0,5 µg/L, microplastics 0,8 mg/L and mixture) on catalase activity in mussels' digestive gland at two and four weeks of exposure are shown in Figures 3.6 and 3.7. In the control tanks mean catalase activity (Units) was 1,49 ± 0,52 at 15 days and 1,10 ± 0,39 at 29 days, in the clarithromycin tanks was 2,09 ± 0,44 at 15 days and 1,94 ± 0,15 at 29 days, in the venlafaxine tanks 2,32 ± 0,57 at 15 days and 1,35 ± 0,44 at 29 days, in the microplastic tanks 1,27± 0,47 at 15 days and 1,78 ± 1,54 at 29 days and in the mixture ones 1,32 ±. 0,24 at 15 days and 0,90 ± 0,31 at 29 days.

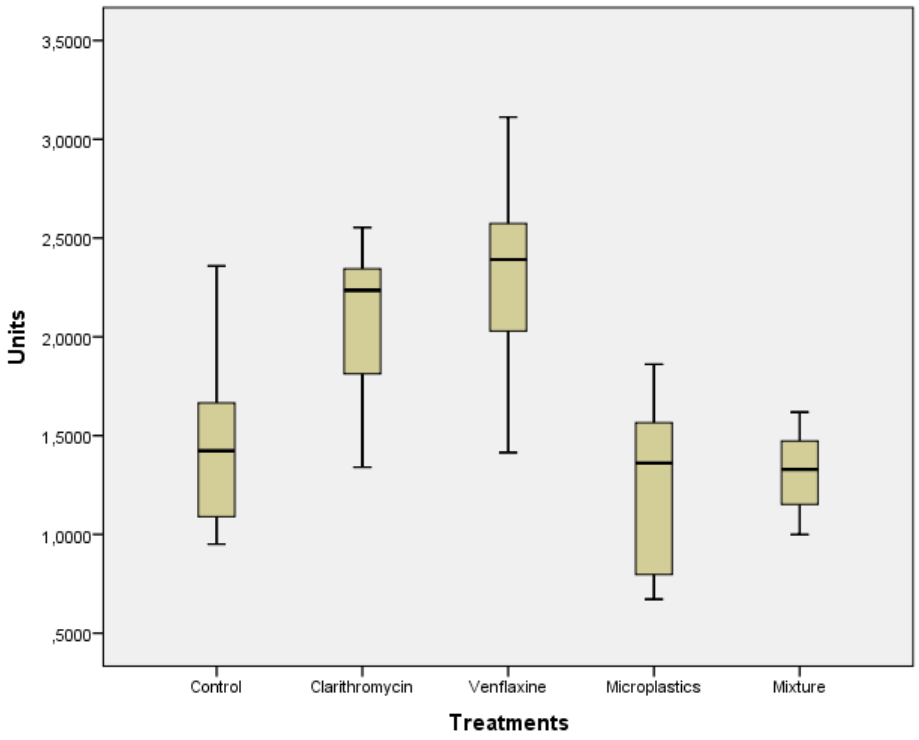

Figure 3.6 Boxplot analysis for catalase for two weeks period. Values are the medians ± standard deviations (n= 6 per treatment).

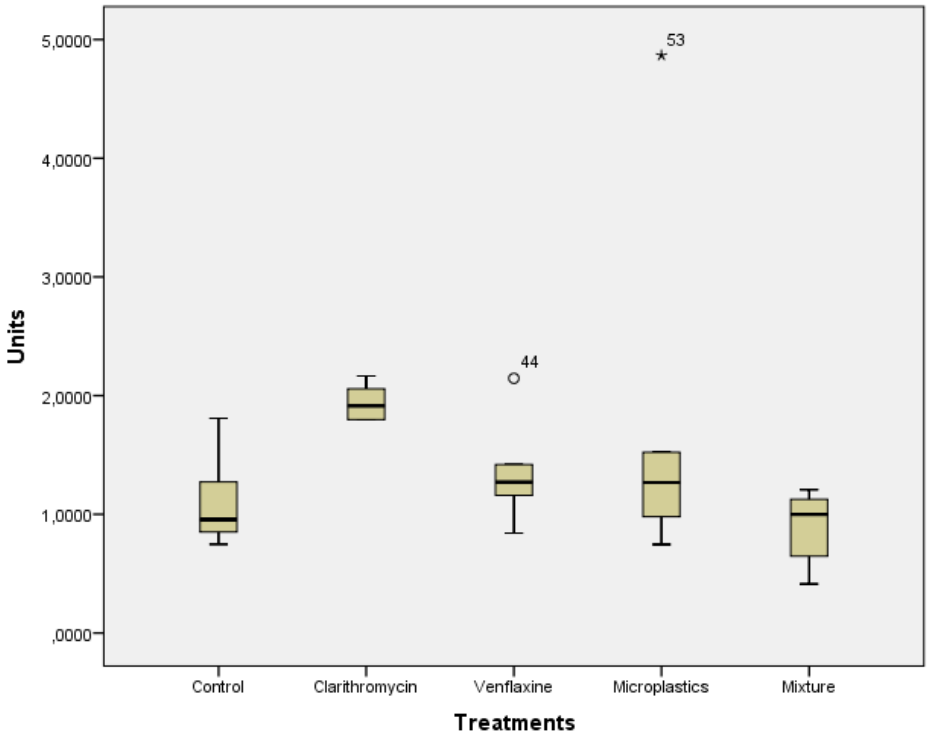

Figure 3.7 Boxplot analysis for catalase for four weeks period. Values are the medians ± standard deviations (n= 6 per treatment). The (*) and the (^{O}) depict the outliers).

Statistical analysis for catalase

The Kolmogorov - Smirnov test was performed to test if data followed normal distribution. The test showed that data derived from a normal population as shown in the Table 3.5.

Table 3.5 Kolmogorov-Smirnov's test of normality.

	Kolmogorov-Smirnov[a]		
	Statistic	df	Sig.
Log10_lnCAT+1	,070	60	,200*

*. This is a lower bound of the true significance.

a. Lilliefors significance correction

The Levene's test was applied to check for equality of error variances for catalase data. The test showed homogeneity of variance as shown in Table 3.6.

Table 3.6 Levene's test of equality of error variances for catalase data.

F	df1	df2	Sig.
1,427	9	50	,202

Since data complied with the assumptions of normality and homogeneity of variance, a univariate analysis was performed. The two way ANOVA showed significant differences in CAT activities among treatments (F= 6,191, P= 0,001) and between sampling periods (F= 5,487, P= 0,023). The interaction of the fore-mentioned factors didn't show any significance (F= 2,213, P= 0,081).

Table 3.7 Two way ANOVA for catalase with treatment and sampling periods as factors.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,319[a]	9	,035	4,345	,000
Intercept	9,247	1	9,247	1131,796	,000
Treatment	,202	4	,051	6,191	,000
Weeks	,045	1	,045	5,487	,023
Treatment * Weeks	,072	4	,018	2,213	,081

a. R Squared = ,439 (Adjusted R Squared = ,338)

Therefore, a multiple comparison test was performed (LSD) that showed significant differences between five pairs of treatments. Significant differences in catalase activity of mussels were found between the control and the clarithromycin (P=0,001) as well as the venlafaxine treatment (P=0,02), between the clarithromycin and the microplastics (P=0,008) as well as the mixture treatment (P=0,001) and finally

venlafaxine and the mixture treatment (P=0,002). Catalase activities of mussels were higher in the clarithromycin and in the venlafaxine than in the control treatment. In addition, in the clarithromycin treatment, catalase activities were higher than in the microplastics and the mixture treatment and finally in the venlafaxine treatment catalase activities were higher than in the mixture.

Where sampling period is concerned, all the treatments showed decreased activities at four weeks with the highest reduction in the venlafaxine treatment.

Table 3.8 Multiple comparisons for catalase (LSD).

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Clarithr	-,124640*	,0369019	,001	-,198759	-,050520
	Venlaf	-,088312*	,0369019	,020	-,162432	-,014193
	Micropl	-,023372	,0369019	,529	-,097492	,050747
	Mixture	,034076	,0369019	,360	-,040044	,108195
Clarithromycin	Control	,124640*	,0369019	,001	,050520	,198759
	Venlaf	,036327	,0369019	,330	-,037792	,110447
	Micropl	,101267*	,0369019	,008	,027148	,175387
	Mixture	,158715*	,0369019	,000	,084596	,232835
Venlafaxine	Control	,088312*	,0369019	,020	,014193	,162432
	Clarithr	-,036327	,0369019	,330	-,110447	,037792
	Micropl	,064940	,0369019	,085	-,009180	,139059
	Mixture	,122388*	,0369019	,002	,048268	,196508
Microplastics	Control	,023372	,0369019	,529	-,050747	,097492
	Clarith	-,101267*	,0369019	,008	-,175387	-,027148
	Venlaf	-,064940	,0369019	,085	-,139059	,009180
	Mixture	,057448	,0369019	,126	-,016671	,131568
Mixture	Control	-,034076	,0369019	,360	-,108195	,040044
	Clarithr	-,158715*	,0369019	,000	-,232835	-,084596
	Venlaf	-,122388*	,0369019	,002	-,196508	-,048268
	Micropl	-,057448	,0369019	,126	-,131568	,016671

3.2.3. Glutathione S Transferase activity

The effect of the four treatments (clarithromycin 1,5µg/L, venlafaxine 0,5 µg/L, microplastics 0,8 mg/L and mixture) on glutathione S transferase activity in mussels' digestive gland at two and four weeks of exposure are shown in Figures 3.8 and 3.9. In the control tanks mean glutathione S transferase activity (nmoles CDNB) was 52,46 ± 9,56 at 15 days and 39,24 ± 4,88 at 29 days, in the clarithromycin tanks was 65,05 ± 13,32 at 15 days and 41,42 ± 4,44 at 29 days, in the venlafaxine tanks 69,10 ± 12,42 at 15 days and 42,23 ± 3,79 at 29 days, in the microplastic tanks 71,62 ± 14,88 at 15 days and 44,08 ± 11,67 at 29 days and in the mixture ones 68,46 ± 10,21 at 15 days and 31,22 ± 6,26 at 29 days.

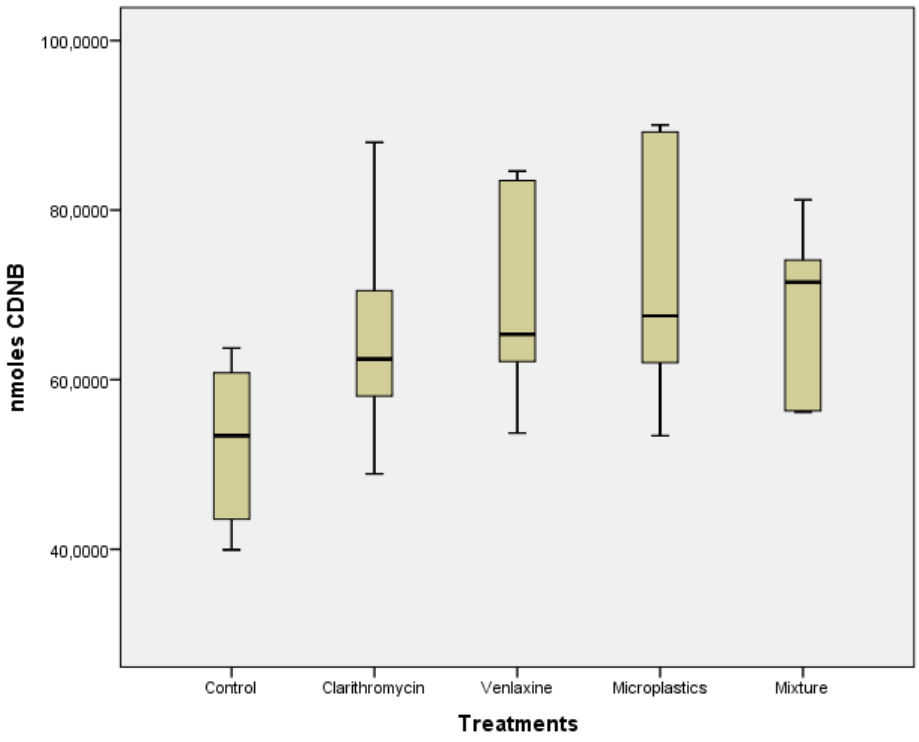

Figure 3.8 Boxplot analysis for glutathione S transferase for two weeks period (n= 6 per treatment The bold horizontal line depicts the median and the stars depict the outlier).

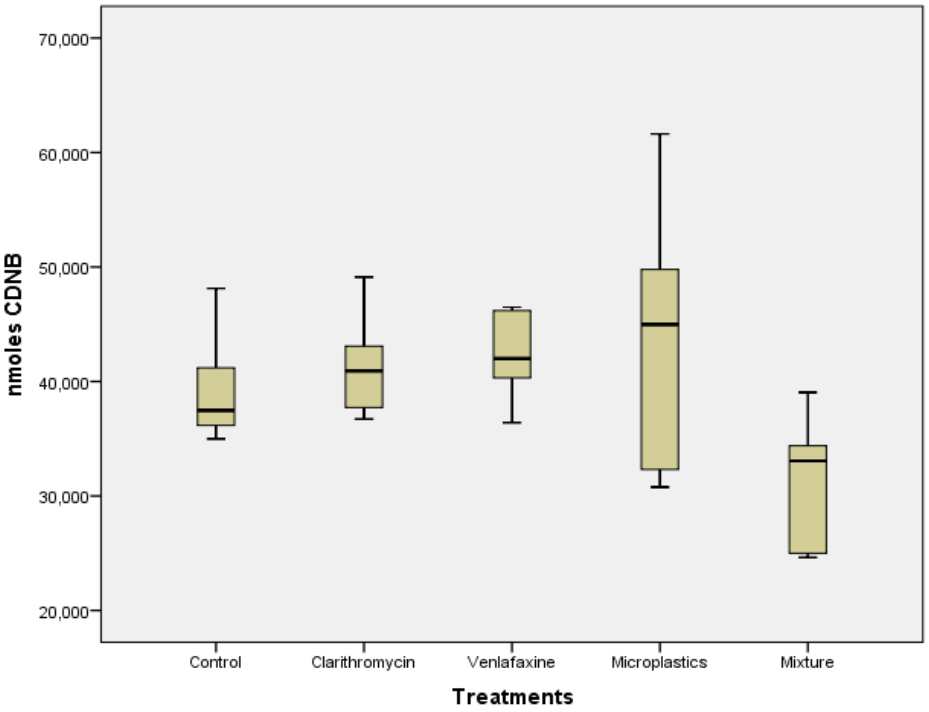

Figure 3.9 Boxplot analysis for glutathione S transferase for four weeks period. Values are the medians ± standard deviations (n= 6 per treatment).

Statistical analysis for Glutathione S Transferase

With the view to testing if data followed a normal distribution, the Kolmogorov - Smirnov test was performed. The test showed that data originated from a normal population as shown in the Table 3.9.

Table 3.9 Kolmogorov-Smirnov's test of normality.

	Kolmogorov-Smirnov[a]		
	Statistic	df	Sig.
Log10_gst+1	,082	60	,200*

*. This is a lower bound of the true significance.

a. Lilliefors significance correction

The Levene's test was applied to check for equality of error variances for glutathione S transferase data. The test showed homogeneity of variance as shown in Table 3.10.

Table 3.10 Levene's test of equality of error variances for glutathione S transferase.

F	df1	df2	Sig.
1,463	9	50	,188

Since data complied with the assumptions of normality and homogeneity of variance, a univariate analysis was performed The two way ANOVA showed significant differences in glutathione S transferase activities between sampling periods (F= 87,928, P= 0,001). The interaction of the fore-mentioned factors didn't show any significance (F= 1,556, P= 0,201) as it happened among the treatments (F= 2,052, P= 0,101).

Table 3.11 Two way ANOVA for glutathione S transferase with treatment and sampling periods as factors.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,819[a]	10	,082	13,688	,000
Intercept	53,321	1	53,321	8913,692	,000
Weeks	,701	2	,351	58,601	,000
Treatment	,075	4	,019	3,143	,022
Weeks * Treatment	,073	4	,018	3,045	,026

a. R Squared = ,736 (Adjusted R Squared = ,683)

As a consequence, a multiple comparison test was performed (LSD) that showed statistical levels of difference between two pairs of treatment. Significant differences in glutathione S transferase activity of mussels were found between the control and the venlafaxine (P=0,032) as the microplastics treatment (P=0,016). Glutathione S

transferase activities of mussels were lower in the control than in the venlafaxine treatment and in control than in the microplastics treatment.

Concerning sampling period, all the treatments showed decreased activities at four weeks with the highest reduction in the mixture treatment.

Table 3.12 Multiple comparisons for glutathione S transferase (LSD).

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Clarithr	-,057170	,0341662	,101	-,125795	,011454
	Venlaf	-,075176*	,0341662	,032	-,143801	-,006551
	Micropl	-,085504*	,0341662	,016	-,154128	-,016879
	Mixture	-,031410	,0341662	,362	-,100035	,037214
Clarithromycin	Control	,057170	,0341662	,101	-,011454	,125795
	Venlaf	-,018006	,0341662	,601	-,086631	,050619
	Micropl	-,028333	,0341662	,411	-,096958	,040291
	Mixture	,025760	,0341662	,454	-,042865	,094385
Venlafaxine	Control	,075176*	,0341662	,032	,006551	,143801
	Clarithr	,018006	,0341662	,601	-,050619	,086631
	Micropl	-,010328	,0341662	,764	-,078952	,058297
	Mixture	,043766	,0341662	,206	-,024859	,112390
Microplastics	Control	,085504*	,0341662	,016	,016879	,154128
	Clarithr	,028333	,0341662	,411	-,040291	,096958
	Venlaf	,010328	,0341662	,764	-,058297	,078952
	Mixture	,054093	,0341662	,120	-,014531	,122718
Mixture	Control	,031410	,0341662	,362	-,037214	,100035
	Clarithr	-,025760	,0341662	,454	-,094385	,042865
	Venlaf	-,043766	,0341662	,206	-,112390	,024859
	Micropl	-,054093	,0341662	,120	-,122718	,014531

4. Discussion

The present study aimed to evaluate the responses of a typical marine bioindicator, the filter feeding *M. galloprovincialis*, towards pharmaceutical compounds and microplastics. The mussels were exposed for 29 days to 1,5μg/L clarithromycin, 0,5 μg/L venlafaxine, 0,8 mg/L microplastics and the mixture of the forementioned under laboratory conditions, and they were investigated for different sublethal responses.

Clarithromycin is widely used as an antibiotic not only in humans but also in animals. It is included in the first watch list which is developed under the Environmental Quality Standards Directive (2008/105/EC) and too little is known about its consequences in seas (Carvalho et al. 2015). The consumption of antidepressants, such as venlafaxine, has increased Greece in the last years because of the economic crisis and they are commonly found in wastewaters (Thomaidis et al. 2016). Venlafaxine has been detected in mussels' tissues from Mediterranean Sea (Bueno et al. 2014, Álvarez-Muñoz et al. 2015). Microplastics have been detected in a variety of aquatic organisms (Wright et al. 2013) and their effects are not well known.

The chosen concentrations of pharmaceuticals were representative of those detected in STP impacted sites and effluents (Kümmerer 2009, Schultz & Furlong 2008) and were considerably lower than EC50/LC50 values on fish, *Daphnia magna* and algae (Thomaidi et al. 2015). The applied concentration of microplastics was higher than those found in the field (Phuong et al. 2016). Mussels' mortality was 5,06 % which is an acceptable percentage. The potential sublethal effects of clarithromycin, venlafaxine, microplastics and the mixture were evaluated through the assessment of three biomarkers. The exposure was held to explore neurotoxicity, oxidative stress and biotransformation effects.

Despite the fact that pharmaceuticals are widely used for human therapy, there aren't many things known about their fate in the aquatic system. It is recognized nowadays that it is not possible to elicit integrated effects for the environment by measuring chemical concentrations of contaminants only since biological measurements indicate the toxic effects to the organisms alone (Livingstone 2001). Recent studies have concentrated in the assessment of impacts of pharmaceuticals via biomarkers (Table 4.1). Mussels are used in many studies on the effects of pharmaceuticals (Prichard & Granek

2016). A summary of relevant studies of mussels' exposure is shown in the Table 4.1 below. To our knowledge, there are no studies concerning clarithromycin and venlafaxine effects on exposed mussels. An in vitro genotoxicity and immunotoxicity study of venlafaxine on mussels' hemocytes has been performed by Lacaze et al. 2015.

Table 4.1 Studies on *Mytilus galloprovincialis* exposed to pharmaceutical compounds adapted from Prichard & Granek 2016 and updated.

In-text Citation	Title	Contaminant	Contaminant Category	Test Organism Collection Location	Response Variable	Study Period	Concentration Range
Boillot et al. 2017	In vivo exposure of marine mussels to carbamazepine and 10-hydroxy-10,11-dihydro-carbamazepine: Bioconcentration and metabolization	Carbamazepine	Antiepileptic	Frontignan, France	bioconcentration factors QuEChERS	7 d	10 µg/L
Bouallegui et al. 2017	Histopathology and analyses of inflammation intensity in the gills of mussels exposed to silver nanoparticles: role of nanoparticle size, exposure time, and uptake pathways	Silver nanoparticles (Ag-nps		Bizerte Lagoon (Northeast Tunisia)	Hematoxyline and eosin (H&E)	3, 6, and 12 h	<50 nm, <100 nm
Cappello et al. 2017	Sex steroids and metabolic responses in mussels *Mytilus galloprovincialis* exposed to drospirenone	Drospirenone	EDC	Ria Formosa lagoon, Portugal	levels of progesterone (P4) and testosterone (T) NMR	7 d	20 ng/J-10 µg/L
Courant et al. 2017	Exposure of marine mussels to diclofenac: modulation of prostaglandin biosynthesis	Diclofenac	Anti-inflammatory drugs	Bouzigues, France	liquid chromatography	3 d	1 and 100 µg/L
Estévez-Calvar et al. 2017	Adverse effects of the SSRI antidepressant sertraline on early life stages of marine invertebrates	Sertraline	Antidepressant	La Spezia, Italy	embryotoxicity	1,2 d	0.01–0.1–1–10–100–1000 µg/L

Luis et al. 2017	Effects of emerging contaminants on neurotransmission and biotransformation in marine organisms - An in vitro approach	Aunps, Carbamazepine, Fluoxetine	Antidepressant, Antiepileptic	Aveiro (Portuga	acetylcholinesterase activity, glutathione S-transferase	15 d	54 ng/L-2,5 mg/L, 20ng/L-1000mg/L
Silva et al. 2017	Uptake, accumulation and metabolization of the antidepressant fluoxetine by *Mytilus galloprovincialis*	Fluoxetine	Antidepressant	Ria Formosa lagoon, Portugal	LC-MS*n histology*	15 d	75 ng/L
Teixeira et al. 2017	Toxic effects of the antihistamine cetirizine in mussel *Mytilus galloprovincialis*	Cetirizine	Antihistamine	Ria de Aveiro, Portugal	ergy-related parameters (glycogen content, GLY; protein content, PROT; electron transport system activity, ETS), and oxidative stress markers (superoxide dismutase activity, SOD; catalase activity, CAT; glutathione S-transferases activity, GSTs; lipid peroxidation levels, LPO; reduced (GSH) and oxidized (GSSG) glutathione content)	28 d	0.3, 3.0, 6.0 and 12.0 µg/L
Matozzo et al 2016	Assessing the Effects of Amoxicillin on Antioxidant Enzyme Activities, Lipid Peroxidation and Protein Carbonyl Content in the Clam *Ruditapes philippinarum* and the Mussel *Mytilus galloprovincialis*	Amoxicillin	Antibiotic	Lagoon of Venice (Italy)	lipid peroxidation levels (LPO) and protein carbonyl content (PCC)	1, 3, 7 d	100, 200 and 400 µg/L
Mezzelani et al. 2016	Transcriptional and cellular effects of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in experimentally exposed mussels, *Mytilus galloprovincialis*	Acetaminophen, diclofenac, ibuprofen, ketoprofen, nimesulide	NSAID	Ancona, Adriatic sea	liquid chromatography	15 d	0.5 µg/L

Banni et al. 2015	Molecular and cellular effects induced in *Mytilus galloprovincialis* treated with oxytetracycline at different temperatures	Temperature, oxytetracycline	Temperature, Antibiotic	NW Adriatic Sea	Lysosomal membrane stability, catalase activity, glutathione S-transferase activity, hsp90, hsp70, hsp27, cAMP, malonedialdehyde accumulation,	4 d	16°C - 24°C, 1 µg/L - 100 µg/L
Franzellitti et al. 2015	A multibiomarker approach to explore interactive effects of propranolol and fluoxetine in marine mussels	Fluoxetine, propranolol	Antidepressant, beta-blocker	NW Adriatic Sea	Fluoxetine tissue concentration, propranolol tissue concentration, lysosomal membrane stability, lysosome/cytoplasm volume ratio, neutral lipids, lipofuscin, catalase activity, glutathione S-transferase activity, DNA damage, CAT mRNA, GST mRNA	7 d	0.3 ng/L
Franzellitti et al. 2014	An exploratory investigation of various modes of action and potential adverse outcomes of fluoxetine in marine mussels	Fluoxetine	Antidepressant	NW Adriatic Sea	Cyclic adenosine monophosphate, protein kinase A activity, ABCB mRNA expression, 5-HT1 mRNA expression, lysosomal membrane stability, lysosome/cytoplasm volume ratio, lipid peroxidation levels, glutathione-s-transferase activity, catalase activity, acetylcholinesterase activity	7 d	0.03 ng/L - 300 ng/L
Gonzalez-Rey et al. 2014a	Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*	Diclofenac	NSAID	Ria Formosa, SE Portugal	Condition index, superoxide dismutase, catalase, glutathione reductase, glutathione-S-transferase, lipid peroxidation levels, acetylcholinesterase activity	15 d	250 ng/L
Gonzalez-Rey et al. 2014b	Effects of active pharmaceutical ingredients mixtures in mussel *Mytilus galloprovincialis*	Ibuprofen + diclofenac + fluoxetine, ibuprofen + diclofenac + fluoxetine + copper	NSAID + NSAID + Antidepressant, NSAID + NSAID + Antidepressant + Metal	Tavira, Portugal	Condition index, superoxide dismutase activity, catalase activity, glutathione reductase activity, glutathione-S-transferase activity, lipid peroxidation, acetylcholinesterase activity, alkali-labile phosphate (vitellogenin determination), gene expression alterations in genes encoding superoxide dismutase, catalase, glutathione reductase, and CYP4YA.	15 d	5 µg/L - 250 µg/L
Koutsogiannaki et al. 2014	Oxidative stress parameters induced by exposure to either cadmium or 17β-estradiol on *Mytilus*	17β-estradiol	EDC	Thessaloniki, Greece	Reactive oxygen species production, lipid peroxidation, protein carbonylation, DNA damage, superoxide dismutase activity,	30 min	25 nM - 5 µM

	galloprovincialis hemocytes. The role of signaling molecules.				glutathione S-transferase activity, catalase activity		
Franzellitti et al. 2013	The mode of action (MOA) approach reveals interactive effects of environmental pharmaceuticals on *Mytilus galloprovincialis*	Propranolol, fluoxetine	Beta-blocker, Antidepressant	NW Adriatic Sea	Cyclic adenosine monophosphate, protein kinase A activity, ABCB mRNA expression, 5-HT mRNA expression	7 d	0.3 ng/L
Gomez et al. 2012	Bioconcentration of two pharmaceuticals (benzodiazepines) and two personal care products (UV filters) in marine mussels (*Mytilus galloprovinialis*) under controlled laboratory conditions	2-ethylhexyl-4-trimethoxycinnamate, Octocrylene, Diazepam, Tetrazepam	UV filter, UV filter, Benzodiazepine, Benzodiazepine	Frontignan, France	Bioconcentration potential	14 d	2 µg/L - 100 µg/L
Gonzalez-Rey et al. 2012	Does non-steroidal anti-inflammatory (NSAID) ibuprofen induce antioxidant stress and endocrine disruption in mussel *Mytilus galloprovincialis?*	Ibuprofen	NSAID	Ria Formosa, SE Portugal	Superoxide dismutase activity, catalase, glutathione reductase, glutathione S-transferase, lipid peroxidation levels, alkali-labile phosphate (vitellogenin evaluation)	15 d	250 ng/L
Franzellitti et al. 2011	The beta-blocker propranolol affects cAMP-dependent signaling and induces the stress response in Mediterranean mussels, *Mytilus galloprovincialis*	Propranolol	Beta-blocker	NW Adriatic Sea	Cyclic adenosine monophosphate, protein kinase A activity, Mussel P-glycoprotein mRNA expression, lysosomal membrane stability, catalase activity, glutathione S-transferase	7 d	0.3 ng/L - 30,000 ng/L
Schmidt et al. 2011	Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus* spp.) and their comparison with standardized toxicity tests	Gemfibrozil, diclofenac	Fibrate, NSAID	West Ireland (Lettermullen, Co. Galway)	Fulton's condition factor, metallothionein, glutathione S-transferase, lipid peroxidation, DNA damage, vitellogenin	1, 4 d	1 µg/L - 1000 µg/L
Solé et al. 2010	Effects on feeding rate and biomarker responses of marine mussels experimentally exposed to propranolol and acetaminophen	Propranolol, acetaminophen	Beta-blocker, NSAID	Trebarwith Strand, England	Feeding rate, phase I carboxylesterase activity, acetylcholinesterase activity, glutathione S-transferase activity, catalase activity, lipid peroxidation, total protein content	10 d	20 µg/L - 200 µg/L

Martin-Diaz et al. 2009	Effects of environmental concentrations of the antiepileptic drugs carbamazepine on biomarkers and cAMP-mediated cell signaling in the mussel *Mytilus galloprovincialis*	Carbamazepine	Antiepileptic	NW Adriatic Sea	Lysosomal membrane stability, accumulation of neutral lipids, accumulation of lipofuscins, lipid peroxidation, glutathione S-transferase, catalase activity, DNA damage, cAMP. PKA activity, expression of MXR-related mRNA	7 d	0.1 µg/L - 10 µg/L
Canesi et al. 2008	Short-term effects of environmentally relevant concentrations of EDC mixtures on *Mytilus galloprovincialis* digestive gland	17b-estradiol, 17α-ethynyl estradiol, Mestranol, Nonylphenol, Nonylphenol monoethoxylate carboxylate, Bisphenol A, Benzophenone	EDC, EDC, EDC, EDC, EDC, EDC, EDC	Gabicce Mare, Italy	Lysosomal membrane stability, accumulation of neutral lipids, accumulation of lipofuscins, catalase activity, GSH transferase, GSSG reductase, lipid peroxidation, phosphofructokinase, pyruvate kinase, protein content, RNA content, malondialdehyde	3 d	0.0177 ng/ml - 177 ng/ml hemolymph
Canesi et al. 2007b	Effects of Triclosan on *Mytilus galloprovincialis* hemocyte function and digestive gland enzyme activities: Possible modes of action on non target organisms	Triclosan	PCP	Cesenatico, Italy	Lysosomal membrane stability evaluated through kinase activity, phagocytosis, lysosomal enzyme release, phosphofructokinase activity, pyruvate kinase activity, GSH transferase, GSSG reductase	1 d	0.29 mg/L - 29 mg/L
Canesi et al. 2007c	Effects of 17β-estradiol on mussel digestive gland	17β-estradiol	EDC	Gabicce Mare, Italy	Lysosomal membrane stability, accumulation of neutral lipids, accumulation of lipofuscins, phosphofructokinase, pyruvate kinase, catalase activity, protein content, MT10 mRNA, MT20 mRNA, p53-like mRNA, Catalase mRNA, Cathepsin L mRNA, 18S mRNA	1 d	5 pmol - 100 pmol

Canesi et al. 2007d	Bisphenol-A alters gene expression and functional parameters in molluscan hepatopancreas	Bisphenol-A	EDC	Gabicce Mare, Italy	MT10 mRNA, MT20 mRNA, p53-like mRNA, Catalase mRNA, Cathepsin L mRNA, 18S mRNA, MeER1 mRNA, MeER2 mRNA, catalase activity, GSH transferase, GSSG reductase, total glutathione content, lysosomal membrane stability	1 d	3 ng/g dw - 60 ng/g dw
Canesi et al. 2007e	Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: In vitro and in vivo studies	17b-estradiol, 17a-ethynyl estradiol, Mestranol, Nonylphenol, Nonylphenol monoethoxyl ate carboxylate, Bisphenol A, Benzopheno ne	EDC, EDC, EDC, EDC, EDC, EDC, EDC	Gabicce Mare, Italy	Lysosomal membrane stability, phagocytosis, lysosomal enzyme release	1 d	0.001 µM - 500 µM
Janer et al. 2005	Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis:* A possible regulating role for steroid acyltransferases	17β-estradiol	EDC	NE Spain	Total testosterone concentrations, total estradiol concentrations, P-450 aromatase activity, Palmoitoyl-CoA:estradiol acyltransferase activity, visual histological analysis	7 d	20 ng/L - 2000 ng/L
Canesi et al. 2004	Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways	Diethylstilbesti Bisphenol-A, 4	EDC, EDC, EDC, EDC, EDC, EDC	Gabicce Mare, Italy	Lysosomal membrane stability, phosphorylation of mitogen activated protein kinases, phosphorylation of signal transducers and activators of transcription	30 min	0 µM - 50 µM

4.1. Acetylcholinesterase activity

AChE activity did not show any statistically significant difference among control and mussels exposed to the two pharmaceuticals and microplastics suggesting absence of neurotoxic effects at the concentrations applied. The higher AChE activities in the mixture treatment indicate interactions among the tested substances but further investigations are needed for their interpretation. For example, the sorption of clarithromycin on microplastic particles could block its uptake by mussels.

Concerning temporal differences, a marked decrease was observed at 29 days of exposure in the clarithromycin, venlafaxine and microplastics treatments suggesting that if periods of exposure were longer, an inhibitory effect on AChE could have been noted. Inhibitory effect on mussels AChE have been reported after exposure to pharmaceuticals. The AChE activity was inhibited by active pharmaceutical ingredients (APIs), such as paracetamol/acetaminophen (23 and 403 μg/ L) in the gills of *M. galloprovincialis* (Solé et al, 2010). However, other studies showed an increase in AChE activity when mixtures of non-steroidal anti-flammatory drugs ibuprofen and diclofenac and selective serotonin reuptake inhibitor, (SSRI) fluoxetine, were given to mussels as it happened at present work. AChE induction is associated with cell apoptosis in various human and mammalian cells possibly because AChE is released after cell membrane disruption (Zhang et al. 2002).

4.2. Catalase activity

The results obtained demonstrated that catalase activity was increased in clarithromycin and venlafaxine tanks compared to controls throughout the exposure. Results suggest that clarithromycin and venlafaxine induce catalase possibly as a defensive protection against oxidative stress. In these two treatments mussels showed significantly higher catalase activities than in microplastics and mixture throughout the experiment. No effect was recorded on catalase activity in microplastics and mixture treatments that could also be explained by sorption of clarithromycin and venlafaxine on microplastic particles which could block their uptake by mussels.

Exposure to antibiotics demonstrates variable effects in catalase of bivalves. Catalase activity was affected slightly from exposure to amoxicillin in either gills or

digestive gland of mussels (Matozzo et al. 2016). The exposure of clams (*R. philippinarum*) to the antibiotic trimethoprim did not significantly affect catalase activities in either gills or digestive gland (Matozzo et al. 2015) while catalase activity increased significantly after 3 and 7 d to exposure of ibuprofen (Gonzalez-Rey and Bebianno 2012).

In accordance with our results, another study clearly demonstrated an increase on CAT activities with the increase of the concentration of the pharmaceutical compound, cetirizine, as a defense mechanism against cetirizine impacts, namely overproduction of reactive oxygen species (ROS) (Teixeira et al. 2017). Similar investigations for mussel exposure, revealed that carbamazepine (0.1 and 10.0 μg/L) for 7 d showed increased activity of CAT in the digestive glands (Martin-Diaz et al. 2009).

On the contrary, *M. galloprovincialis* exposed to different pharmaceuticals during 24 days showed a different response of catalase towards each drug. There were not detected any changes in enzyme activity when mussels were exposed to acetaminophen, diclofenac and ibuprofen. The significant decreases detected when organisms were exposed to ketoprofen and nimesulide (Mezzelani et al. 2016). Concerning microplastics, a similar study in mussels exposed to PS microparticles, showed decrease in catalase activities and modulation of cellular oxidative balance (Paul-Pont et al. 2016).

4.3. Glutathione S Transferase activity

The measurements of the glutathione S transferase activity showed higher values in venlafaxine and in microplastics treatments than in controls treatments. These results suggest that venlafaxine and microplastics exposure induced biotransformation and/ or the antioxidant defense mechanism.

Another study on mussels showed that the simultaneous exposure of pharmaceuticals and citrate coated gold nanoparticles (cAuNPs) induce GST activity when this is compared to the pharmaceuticals individual effect, though only significantly for carbamazepine with 54 ng/ L cAuNPs and fluoxetine with 54 ng/ L and cAuNPs 2,5 mg/ L (Luis et al. 2016).

Other surveys in mussels, explored the presence of fibrates in aquatic environment. Fibrates, as bezafibrate and gemfibrozil, increased GST and GSR activities,

as well as total glutathione content even though at different concentrations, indicating a significant effect on GSH metabolism.

In accordance with our results, Paul- Pont et al. 2016 also found a increase in GST activities in mussels exposed to PS microparticles for a period of time 7 days.

4.4. Conclusions

The present work showed effects of clarithromycin and venlafaxine exposure on catalase activity of mussels and effects of venlafaxine and polysterene microplastics on glutathione S transferase activity, suggesting induction of antioxidant defense and/ or biotransformation.

Overall, our results support the hypothesis that human pharmaceuticals can lead to significant effects on non-target species and that bivalve mollusks may represent sensitive organisms for the action of these compounds in the environment (Canesi et al. 2007a).

4.5. Future prospects

A more integrated study of the exposure of pharmaceutical compounds and microplastics could include the use of more biomarkers, such as vitellogenin (biomarker of endocrine disruption) or lysosomal stability (biomarker of general stress), with the aspect to obtain results on a more holistic determination of mussels' health.

Chemical analysis of the pharmaceuticals in the mussels' tissues would contribute to the interpretation of our results.

Further studies exposing bivalves for a longer period of time are needed to better evaluate the risk posed by those treatments (Matozzo et al. 2016) and to determine whether the organisms respond to the prolonged exposure more intensively.

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