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Impairment of haemocytes in the Mediterranean mussel (*Mytilus galloprovincialis*) exposed to glyphosate

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Abstract

This study analysed the effects of glyphosate, a systemic, non-selective herbicide that is commonly used as a plant growth regulator, on haemocyte in the Mediterranean mussel, *Mytilus galloprovincialis*. Three treatments where performed using glyphosate alone, its degradation product aminomethylphosphonic acid (AMPA), and a combination of both. Basal transport activity was assessed using the model substrate rhodamine 123 and specific inhibitors for the MXR-related transporter P-glycoprotein (ABCB mRNA). Haemocytes exposed to glyphosate (0.1 and 1 µg/L) showed significant reduction of total Multixenobiotic resistance (MXR) efflux activity that was not apparently related to transcriptional expression of genes encoding P-glycoprotein. Oppositely, both samples exposed to glyphosate and AMPA separately, showed a decrease accumulation of Rho123, suggesting no alteration of the MXR efflux activity. At these same concentrations, transcriptional profiles of 6 genes underlying key biological functions in mussels and potential targets of adverse effects of glyphosate were analysed. These specific genes were analysed to study the immune responses (lysozyme, LYS; mytilin b, MYTLb; myticin c, MYTCc), and lysosomal system (cathepsin L, CTSL; ß-glucuronidase, GUSB; hexosaminidase, HEX). Results indicated a down regulation of the lysosomal genes, suggesting that this contaminant may cause a decrease in lysosomal membrane stability and loss of lysosomal functionality. Furthermore, an up regulation of the immune genes might indicate that it is a response to the microbial alterations that occur as a result of glyphosate exposure. Overall, the data obtained highlighted the potential for these pharmaceuticals to cause immunotoxicological effects as part of their adverse outcomes in mussels.

Key words: Haemocyte, ABC transporter, Marine mussels, Glyphosate, Multixenobiotic resistance (MXR)

Resumen

Este estudio analiza los efectos del glifosato, un herbicida sistémico, no selectivo que se utiliza como regulador del crecimiento de las plantas, sobre los hemocitos del mejillón mediterráneo. Se realizaron tres tratamientos utilizando glifosato, su producto de degradación ácido aminometilfosfónico (AMPA) y una combinación de ambos. La actividad de transporte basal se evaluó utilizando la rodamina 123 e inhibidores específicos para el transportador P-glicoproteína relacionado con MXR (ARNm de ABCB). Los hemocitos expuestos a glifosato (0,1 y 1 µg/L) mostraron una reducción de la actividad de expulsión del sistema de resistencia multixenobiótica (MXR) no relacionada con la expresión transcripcional de genes que codifican la P-glicoproteína. Ambas muestras expuestas al glifosato y a AMPA por separado, mostraron una disminución de la acumulación de Rho123, lo que sugiere que no hay alteración de la actividad de expulsión del MXR. También se analizaron los perfiles transcripcionales de 6 genes relacionados con las funciones biológicas de los mejillones y los posibles objetivos del glifosato. Estos genes se analizaron para estudiar las respuestas inmunológicas (lisozima, LYS; mytilin b, MYTLb; myticin c, MYTCc) y el sistema lisosomal (catepsina L, CTSL; ß-glucuronidasa, GUSB; hexosaminidasa, HEX). Los resultados indicaron una regulación a la baja de los genes lisosomales, esto puede causar una disminución en la estabilidad de la membrana lisosomal y pérdida de la funcionalidad lisosomal. En los genes inmunológicos hubo una regulación positiva en respuesta a las alteraciones microbianas causadas por el glifosato. En general, destaca el potencial del contaminante para causar efectos inmunotoxicológicos en los mejillones.

Palabras clave: Hemocitos, Transportador ABC, Mejillones marinos, Glifosato, Resistencia multixenobiótica (MXR).

1. Introduction

1.1. Mytilus galloprovincialis

Mussels of the genus *Mytilus* are among the most common marine molluscs and constitute a fundamental element in the ecology of coastal waters (Azizi *et al.*, 2018). *Mytilus galloprovincialis* (Lamarck, 1819) is characterised by the shell, being elongated, somewhat triangular and wide. It also has asymmetric valves, pointed at one end, with the hinge somewhat curved, and the other side flattened and round. The dorsal edge is straight and the external surface has very fine lines of growth. The shell is bright purplish black on the outside and pearly bluish grey on the inside. It can grow up to 15 cm in length when grown in rafts, but the ones on rocks tend to be smaller. All this features can be seen in the figure 1.

Figure 1. *M. galloprovincialis* shell, dorsal view on the left and ventral view on the right (ICTIOTERM, 2019).

M. galloprovincialis can easily be confused with *M. edulis* since they are very similar. In figure 2, it can be seen a diagram of the anatomy of *M. edulis* that resembles the one in *M. galloprovincialis.*

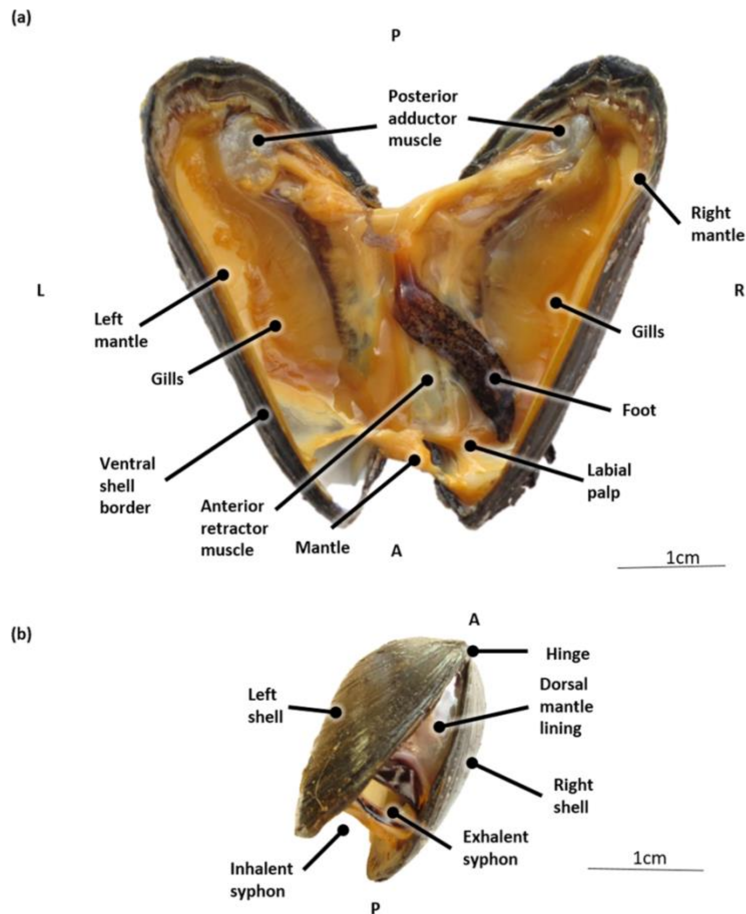

Figure 2. Anatomy of the *Mytilus edulis*. (a) Ventral view. (b) Oblique dorsal view (Eggermont *et al.*, 2020).

M. galloprovincialis, is distributed in the temperate waters of the Mediterranean, as can be seen in figure 3. *M. galloprovincialis* grows on the surface of hard substrata. Salinity, oxygen saturation, temperature, amount of food, illumination and exposure to sea fluctuation, as well as the input of terrigenic materials, all limit its ambient environment. It normally inhabits new areas from January to June and its growth is at its peak from June to September. The most rapid growth of the shell occurs during the summer months, when temperatures range from 22 to 23 degrees Celsius. It prefers areas with little fluctuation in sea level, such as the river mouths.

Figure 3. *Mytilus galloprovincialis* distribution in the Mediterranean (FAO, 2016).

In Italy, the cultivation of this marine mollusc is of great economic importance. Every year, this mollusc is harvested in amount higher than 170,000 tons, which represents more than 75% of the total shellfish production in Italy (Saroglia, Cecchini and Terova, 2000).

Marine bivalve *Mytilus galloprovincialis*, as many other bivalves, has gills, which are two pairs of broad plates composed by a large number of parallel filaments that filter food particles from the water. A 5 cm long mussel can filter up to 5 litters of seawater an hour. Digestion occurs in the digestive gland (greenish-brown in colour) located in the centre of the body. Mussels feed on phytoplankton and organic matter (FAO, 2009; Murgarella *et al.*, 2016).

This alimentation by filtration exposes the organism to many pollutants in seawater, causing them to accumulate and concentrate them, particularly those which are particulate or are associated with particles (Stabili, Acquaviva and Cavallo, 2005). Furthermore, coastal water environments, natural habitat of bivalves, are heavily impacted by fluctuating biotic and abiotic parameters and anthropogenic pollution. This makes the interaction between immune and stress responses in these organisms essential for survival under harsh conditions (Adamo, 2008).

In the North Adriatic Sea, Italy, the production, harvesting and commercialization of bivalve molluscs is regulated by the EC directive 79/923. This directive defines the classification of the waters in which the mussels grow and the EC directive 91/942 states the safety standards for live mollusc sale (Vernocchi *et al.*, 2007).

1.2. Emerging Contaminants (ECs)

Emerging contaminants (ECs) are pollutants that are now uncontrolled but might be regulated in the future based on research into their possible health impacts and data on their incidence (Barceló, Eljarrat and Petrovic, 2002). Because these substances are continually released into the environment at a slightly rising pace (generating "pseudo-persistence"), they do not need to be persistent to cause harm to many organisms (Taheran *et al.*, 2018). Furthermore, the absence of effective and conventional methods for determining emerging pollutants such as pesticides is due to the complexity of matrices and the fact that they are present at extremely low concentrations. The gathering of data on the incidence, pathways, ecotoxicology, and risk assessment of new pollutants has been complicated by this significant analytical problem (Alexander, Hai and Al-aboud, 2012; Dosis *et al.*, 2017; Schmidt, 2018).

Coastal marine ecosystems are biologically important and highly productive ecosystems that provide a variety of natural and socioeconomic resources. Over the last two decades, the exponential population growth along the coast has substantially increased human exploitation of marine resources, resulting in habitat loss, biodiversity depletion, and water contamination from a wide range of anthropogenic substances (European Commission, no date).

A broad range of contaminants, mainly from industrial or agricultural sources, has been regulated in the European Community, and environmental quality standards have been established for 33 priority substances under the Water Framework Directive (WFD) (2000/60/EC). In 2015, the contaminant glyphosate was included in the WFD “Watch List” of possible priority substances (Carvalho, Ceriani and Ippolito, 2015).

1.3. Pesticides residues in the marine environment

Pesticides are chemicals that are used to control pests such as insects, weeds in the water, and plant diseases. Pesticides, do not come without drawbacks, some have a long environmental persistence and are created in significant quantities (up to a million tons per year), to the point where they are now measurable substances in sea water, sediments, and biota (Ernst, 1980; Helfrich *et al.*, 2009).

1.3.1. The interaction between marine organisms and pesticides: bioaccumulation and effects

Because aquatic creatures interact in the food chain, understanding their predisposition to bioconcentrate pesticide residues in water and via dietary exposure is critical when assessing real-world pesticide effects. Bioconcentration is the term used to describe the process by which pesticides enter organisms directly from water, either through the gills or epithelial tissues. Bioaccumulation, on the other hand, takes into account the effect of dietary absorption through food consumption or the ingestion of bottom sediments (Katagi, 2010).

1.3.2. Glyphosate

Glyphosate ($C_3H_8NO_5P$) has the chemical structure shown in figure 4 in 2D (PubChem, 2021).

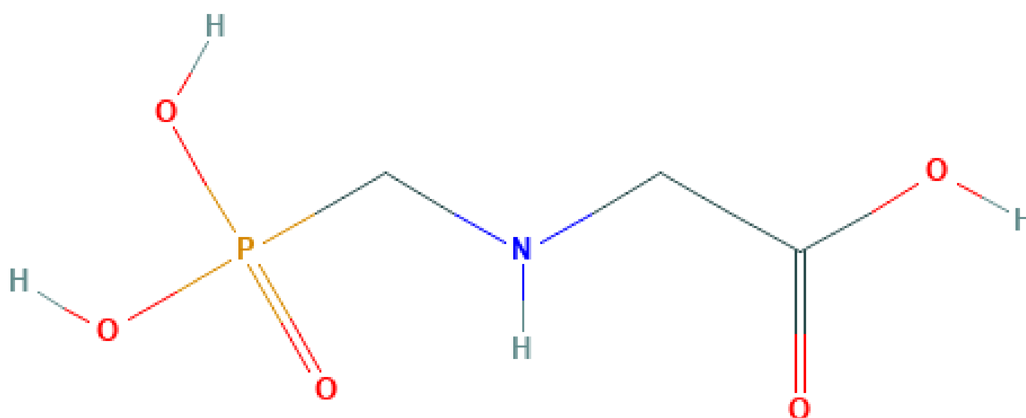

Figure 4. Glyphosate chemical structure represented in 2D (PubChem, 2021).

Concerns about the effect of glyphosate (N-phosphonomethylglycine) on non-target aquatic organisms have grown in recent years (Solomon *et al.*, 2003). Glyphosate is a systemic, non-selective, broad-spectrum herbicide that is commonly used as a plant growth regulator. As such, glyphosate inhibits protein production in plants by affecting the synthesis of essential aromatic amino acids (Hatzios, 1994; Franz, Mao and Sikorski, 1997) . Due to its efficacy, this compound is used in a variety of commercial formulations (de Brito Rodrigues *et al.*, 2017). After application a significant amount of glyphosate can settle on the ground, where it can be degraded or leached (Rashid *et al.*, 2013).

Glyphosate use has raised to 600–750 thousand tons per year worldwide, with an estimated increase to 740–920 thousand tons per year by 2025 (Maggi *et al.*, 2019). Because of the inert C-P linkage in the molecule, glyphosate is quite resistant to

degradation (Chekan, Cogan and Nair, 2016). Being able to persist in both freshwater (7–142 days) and seawater (267–315 days in aphotic condition at 25 ºC and 31 ºC, respectively) (Annett, Habibi and Hontela, 2014; Mercurio *et al.*, 2014). Nevertheless, glyphosate can be degraded by bacteria into aminomethylphosphonic acid (AMPA) or sarcosine (Mamy, Barriuso and Gabrielle, 2005; Borggaard and Gimsing, 2008).

As a result, glyphosate and AMPA may also be found in the environment together (Matozzo *et al.*, 2019). According to a previous report, AMPA primarily affected haemocyte parameters in *M. galloprovincialis* (Matozzo *et al.*, 2018) this lead to the disruption of genes involved in apoptosis, immune response, energy metabolism, Ca2+ homeostasis, cell signalling and endoplasmic reticulum stress response (Milan *et al.*, 2018).

On previous studies glyphosate was found in 39.7% of Italian surface water samples, with 25.2 % of those exceeding the annual environmental quality requirement (SQA-MA) for rivers, as set by current legislation (ISPRA, 2014).

While glyphosate's toxicity to fish and mammals is low, the formulated products are frequently more toxic than glyphosate, and concerns have been raised about the formulated products' long-term effects on human and animal diseases, especially cancer and mental disorders (Swanson *et al.*, 2014; R. Mesnage *et al.*, 2015; Robin Mesnage *et al.*, 2015; Fortes *et al.*, 2016). Previous studies suggests that, in addition to direct toxicity on the host's physiology, changes in the host's microbial community must also be considered (Iori *et al.*, 2020).

Effects of glyphosate in mussels

Previous research found that glyphosate and AMPA (alone or in combination) reduced total haemocyte count (THC) in mussels at the cellular stage. As a result, they hypothesized that this decrease in bivalves observed after exposure to pollutants was due to increased haemocyte mobilization from the haemolymph into peripheral tissues (Matozzo *et al.*, 2019). They also hypothesized that treated mussels' haemocyte proliferation increased to compensate for the reduction in THC, as previously indicated for AMPA-exposed mussels (Matozzo *et al.*, 2018). Overall, this research suggested that the combination of glyphosate and AMPA may affect haemocyte membrane stability, similar to what has been found previously when studying individual compounds (Matozzo *et al.*, 2019).

Furthermore, RNA-seq analyses revealed a transient and time-dependent transcriptional response to each treatment in other studies. Similarly, they suggested that the majority of transcriptional shifts in AMPA and GLY-exposed mussels is treatment-specific, while a cumulative effect occurs in MIX-exposed mussels. The majority of the disrupted molecular pathways pointed to similar toxicity mechanisms for AMPA and GLY. Gene expression profiling of mussels exposed to GLY and AMPA reveals changes in several primary molecular pathways and biological processes, indicating both extensive compensatory responses and acute toxicity. The resulting disruption of host physiological homeostasis may have favored changes in the host microbiota, resulting in the spread of potentially pathogenic opportunists like Vibrio species (Iori *et al.*, 2020).

Effects of glyphosate on other organisms

Many laboratory studies have shown that glyphosate can be absorbed in the human and mammalian gastro-intestinal tract, as well as by inhalation, ingestion, and dermal touch. Moreover, high doses of glyphosate have been used in a number of experiments on mice and rats. The substance induces growth retardation, kidney damage, liver enlargement and inflammation and gastric disease (Williams, Kroes and Munro, 2000; Mercurio *et al.*, 2014; Bailey *et al.*, 2018). In addition, glyphosate is toxic to aquatic plant species and affects critical functions such as photosynthesis, respiration, and the synthesis of essential aromatic amino acids (Torretta *et al.*, 2018).

Increased glyphosate use has been linked to a number of human diseases, including different types of cancer and mental illnesses including ADHD, autism, Alzheimer's, and Parkinson's disease (Jayasumana, Gunatilake and Senanayake, 2014; Swanson *et al.*, 2014; R. Mesnage *et al.*, 2015; Fluegge and Fluegge, 2016; Fortes *et al.*, 2016). Increases in pig infertility and malformation were linked to glyphosate levels in the liver and kidneys, as well as residues in the feed (Krüger, Schrödl and Pedersen, 2014; Philipp Schledorn, 2014).

Thus, glyphosate can have hormonal effects and reduce fertility at low concentrations, at high doses, various other organs may be affected, eventually leading to death (Van Bruggen *et al.*, 2018).

Chronic exposure of gold fish (*Carassius auratus*) to relatively low levels of glyphosate disrupted metabolism in different tissues, resulting in overproduction of ROS and oxidative stress, close to the effects of glyphosate on terrestrial animals (Li *et al.*, 2017).

While conclusions about glyphosate's potential health effects are still discussed, studies show that enough new data about the chronic toxic effects of formulated products on aquatic and terrestrial organisms, as well as humans, has accumulated to warrant reconsidering the tolerable residue levels of glyphosate and AMPA in plants and animal products, as well as the environment (Van Bruggen *et al.*, 2018).

1.4. Biomarkers of environmental stress

Various biomonitoring techniques have been established over the last few decades to track and measure the negative effect of pollutants on marine and estuarine habitats. The monitoring systems employ a large number of bioindicators or biomarkers known as "sentinel organisms" to identify temporal and spatial variations in chemical contaminants and contribute to the understanding of marine pollution patterns (Rodil *et al.*, 2019).

A pollutant stress situation, such as xenobiotic-induced changes in aquatic organism fitness, is caused by a series of biological impacts, each of which may theoretically be utilized as a biomarker (van der Oost, Beyer and Vermeulen, 2003). For biological parameters to be used in this way, however, technical and practical characteristics are required, such as effect reliability, cost-effectiveness, sensitivity to low levels of pollutants, and well-characterized background levels or dose-response profiles (Handy, Galloway and Depledge, 2003). Because of the sensitivity and speed with which xenobiotics cause changes in biomarkers, they can be used to assess harmful effects at lower doses and shorter exposure times than those required to elicit affects at higher hierarchical levels (Moore, Icarus Allen and McVeigh, 2006). In surveys requiring a rapid and sensitive assessment of the environmental quality of contaminated areas, such as oil spill incidents, biomarkers can be used to preliminarily establish cause- or dose-effect correlations (Viarengo *et al.*, 2007).

Biomarkers can be classified into three groups based on their toxicological significance (van der Oost, Beyer and Vermeulen, 2003): biomarkers of effect, biomarkers of exposure and susceptibility biomarkers. Biomarkers of effects are biochemical, cellular, or physiological parameters that reflect the fitness of the organisms being studied. Cellular or subcellular functions, metabolic disorders, pro-oxidant, and histological changes are some of them. By other hand, biomarkers of exposure are the result of a xenobiotic agent's interaction with metabolites or certain physiological and cellular processes. Finally, susceptibility biomarkers are a measure of an organism's ability to

cope with a xenobiotic stimulus, including functional alterations in genetic characteristics implicated in toxicant interactions.

Notably, the parameters of oxidative stress, lysosomal toxicity, and genotoxicity were identified as relevant outputs for the classification of the pollution impacts of marine contaminants and the implementation of the quality requirements stated in Descriptor 8 of the MSFD (Law *et al.*, 2010; Acom, 2011). Since the early 2000s, several of those biomarker-based biomonitoring studies have been done in the marine environment, revealing that a variety of anthropogenic activities can trigger sub-lethal toxicity pathways in the exposed biota (Orbea *et al.*, 2006; Turja *et al.*, 2014).

The Mussel Expert System (MES), is a good example of computer methods used for biomarker effects classification (Dagnino *et al.*, 2007). The MES is built on a collection of principles obtained from data on biomarker responses to natural and pollutant-induced stress in marine mussels. The level of biological organization, biological importance, mutual interdependence, and qualitative trends in a stress gradient are all included in the integration of parameters. In biomonitoring studies, the application of the MES demonstrated suitable for providing a clear indicator of the degree of stress syndrome generated by pollutants (Shaw *et al.*, 2011), and it has been cited as a potential way to assess the dangers of ECs on coastal habitats (Franzellitti *et al.*, 2015).

1.4.1. The use of mussels as model organisms for biomarker-based investigations

Because of their ability to concentrate many environmental pollutants, bivalves, especially mussels, have been widely used as pollution indicators. The basic assumption is that these species can have a reliable time-integrated value for determining the levels of pollutants in the coastal environment (Farrington *et al.*, 2016). Additionally, its broad geographic distribution, sedentary lifestyle, filter feeding activity, and tolerance for a wide range of environmental conditions (Viarengo *et al.*, 2007; Mohamed *et al.*, 2014), are some of the reasons for why the genus *Mytilus* has been widely used as a sentinel organism for marine pollution biomonitoring (Brooks *et al.*, 2015; Benali *et al.*, 2017).

Furthermore, low decontamination kinetics, easiness of detection and selection of samples, and enough tissue for chemical analysis are also important considerations as to why mussels are one of the most suitable indicator organisms (Kristan *et al.*, 2014).

Even so, *Mytilus* calcium carbonate shells may be an interesting substitute for soft tissue in mussel watch programs around the world for heavy metal pollution biomonitoring (Kandǔca, Medakovíc and Hamer, 2011).

In this thesis, a set of molecular biomarkers of environmental stress has been analysed in the Mediterranean mussel *Mytilus galloprovincialis* exposed in laboratory to environmentally relevant concentrations of glyphosate.

1.4.2. Haemocytes as a biomarker

Marine bivalves do not have an immune system instead they have chemicophysical barriers which act as obstacles to outside invasion, such as, external skeletons, cuticles or mucus. Furthermore, bivalve molluscs have an "open" circulatory system in which the blood, or haemolymph, passes out of the open ends of arteries and bathes all of the organs before returning to the heart via sinuses and respiratory systems in bivalve molluscs (gills). Haemolymph contains a variety of proteins, including soluble lectins, lysosomal enzymes, and antimicrobial peptides. These components can be used to trigger a wide range of defence mechanisms. Haemocyte cells are responsible for some vital immunity process (Canesi *et al.*, 2002).

Haemocyte immune function is now regarded as one of the most essential for commercially cultured animals. These cells, whose cytoplasm contains granules of various colour, size, and number, are widely acknowledged to play an important role in bivalves' cellular immunity. These cells can phagocytize microbial pathogens and create superoxide radicals and other reactive oxygen species, both of which contribute to intracellular killing (Hine, 1999; Parrino *et al.*, 2019). Therefore, this cells can be used to explore the effects of pollutants on the mussels immunity, as well as to consider the critical function of defensive mechanisms activated in these cells as a result of immunotoxicological reactions (Franzellitti *et al.*, 2016).

Biomarkers employed in this thesis encompass parameters of multixenobiotic resistance activity (ABCB encoding the P-glycoprotein, Pgp), immune responses (genes encoding lysozyme, LYS; mytilin b, MYTLb; myticin c, MYTCc), and lysosomal system (genes encoding cathepsin L, CTSL; ß-glucuronidase, GUSB; hexosaminidase, HEX).

Multixenobiotic Resistance mechanism

The Multixenobiotic Resistance mechanism (MXR), can be found in aquatic organism and it represents a defence cell system (Kurelec, 1992; Smital *et al.*, 2004). The activity of MXR, is mediated by transmembrane proteins known as ATP Binding Cassette (ABC) transport proteins. This ABC transport proteins, bind and hydrolase ATP, aiming to extract out of cells endogenous and exogenous substrates, this is a crucial step, since it limits their accumulation and potential toxicity (Szakács *et al.*, 2008).

Previous research has shown that both chemical and physical stressors activate the MXR system in Mediterranean mussels, and that MXR protein expression differs significantly among species living in different levels of contamination (Smital *et al.*, 2000). Consequently, one of the molecular processes improving stress tolerance in animals living in polluted environments is the MXR response (Minier *et al.*, 2000; Bielen *et al.*, 2016).

As a part of the MXR, two key transporters can be found, the P-glycoprotein (P-gp) and the Multidrug resistance-related protein (Mrp). P-gp primarily transports unmodified xenobiotics and is designed to aid in phase 0 of defense, on the other hand, Mrp is mostly made up of direct products of phases I and II metabolism. Lysosomal system is also known to have a predominant contribution to haemocyte protection (Svensson, Särngren and Förlin, 2003; Franzellitti *et al.*, 2016)

P-glycoprotein

P-gp works as an energy-dependent pump that transports a wide range of structurally and functionally different substrates. Furthermore, they prevent endogenous metabolites, phospholipids, and xenobiotics from accumulating in exposed animals and cell communities. Proteins that are similar to P-gp have been found in a number of marine species. Natural products and anthropogenic pollutants present in the aquatic ecosystem tend to be substrates and inducers of aquatic organisms multixenobiotic resistance transporter. These findings indicate that P-gp activity, in addition to normal cell function, can play a role in the relative hardiness of aquatic species exposed to xenobiotics (Bard, 2000). In *M. galloprovincialis,* P-gp is encoded by the ABCB gene (Franzellitti and Fabbri, 2006), it has also been proven that the induction of this protein, in mussels, is in response to a wide range of chemical and physical stressors, including, pesticides, as well as temperature or salinity variations (Franzellitti *et al.*, 2016).

Multidrug resistance-related protein

The Multidrug resistance-related protein (Mrp) encoded by the ABCC gene is another ABC transporter found in mussels (Franzellitti and Fabbri, 2006). Mrp substrates are primarily glutathione, glucuronate, or sulphate water soluble conjugates, which are direct products of phases I and II metabolism. As a result, Mrp transporters are referred to as phase III biotransformation processes (Leslie, Deeley and Cole, 2005), and they are found in abundance in exchange tissues such as blood vessels and the gastrointestinal epithelium, where they enable conjugated waste products to be excreted (Leslie, Deeley and Cole, 2005; Deeley, Westlake and Cole, 2006). Mrps are also involved in maintaining cell redox status homeostasis under physiological and stress conditions since they are glutathione-dependent transporters (Deeley, Westlake and Cole, 2006).

Lysosomal system

To conclude, lysosomal responses are important because damaging lysosomal reactions commonly precede cell and tissue diseases, lysosomal-vacuolar system responses may give a solution to the question of predictive biomarkers. Pollutant exposure, for example, has been linked to lysosomal perturbations, which have been routinely employed as early markers of harmful effects (Moore, 2002; Galloway *et al.*, 2004; Moore *et al.*, 2004). As a result, lysosomal function can be utilized to detect reactions to environmental stress in a variety of animals, including annelids, molluscs, crustaceans, and fish (Cajaraville *et al.*, 2000; Köhler, Wahl and Söffker, 2002; Wang, Ade and Ericano, 2002; Galloway *et al.*, 2004; Hankard *et al.*, 2004). Lysosomes are multifunctional cellular organelles found in nearly every cell of eukaryotic species, from yeast to humans. The destruction of superfluous or damaged organelles (e.g., mitochondria and endoplasmic reticulum) and longer-lived proteins as part of autophagic cellular turnover is one of their functions in the cellular economy (Klionsky and Emr, 2000). Enhanced sequestration and autophagy of organelles and proteins are two lysosomal mechanisms involved in normal physiological responses as well as numerous cell injury and disease processes (Klionsky and Emr, 2000; Moore, 2002).

2. Aims of the study

Glyphosate is a common pesticide used in agriculture, which causes it to accumulate in the soil and eventually reach inland water resources. Leading to ultimately accumulate in the marine environment.

Marine mussels are an appropriate creature to study the effects of anthropogenic pollution since they are sessile and have a good filter feeding mechanism. Mussels' habitat in coastal environments is generally sensitive to anthropogenic pollutants, allowing them to be exposed to these toxins more than other marine creatures. As a result, we used Mediterranean mussels (*Mytilus galloprovincialis*) to study the physiological reactions of haemocytes, which serve as the mussel's principal biological barrier against environmental stressors.

Therefore, the main objective of this study is to see whether glyphosate and AMPA will impair haemocytes in *M. galloprovincialis*, causing physiological disturbances in these immune-relevant cells or not. To achieve the main goal, a set of specific objectives were defined as follows:

- To study the most stable reference gene.
- To investigate the effects of glyphosate and AMPA on MXR activity and ABCB mRNA expression.
- Analysis of the effects of glyphosate and AMPA on gene transcription.

3. Materials and methods

3.1. Animal handling and tissues collection

Mediterranean mussels (*Mytilus galloprovincialis*) of commercial size (4-6 cm in length) were harvested from a government-certified mussel farm (Cooperativa Copr.al.mo, Cesenatico, Italy).

The cultivation of *Mytilus galloprovincialis* in this farm is extensive, as well as in all the countries where it is carried out. This means that, young mussels are harvested from the sea and can be grown on suspended ropes. These ropes, which are covered with mussel seeds held in place with nylon nets, are suspended either from rafts, or wooden frames, or from longlines with floating plastic buoys (FAO, 2009).

They were transported to the lab, where they were kept in seawater tanks one day for acclimation, as shown in figure 5. The water in aquaria had the following conditions, continuous aeration (> 90% oxygen saturation), 35-psu artificial sterile seawater (ASW) at a temperature of 16°C.

Figure 5. Mussels in the tanks for acclimation.

Using a sterile 1 mL syringe, haemolymph was collected from the posterior adductor muscle and pooled to get the total volume required for each experiment. This is shown

in figure 6. A total of 50 mussels were used in this experiment (100 total mussels for the whole 3 experimental trials). Fresh haemolymph was immediately used for haemocyte treatments for MXR activity assays and RNA extraction.

Figure 6. Extraction of haemolymph with a sterile 1mL syringe.

3.2. Chemicals

Rhodamine 123 (Rho123) was purchased from Sigma Aldrich (Milan, Italy). The iScript supermix and iTaq Universal master mix with ROX were from Biorad Laboratories (Milan, Italy). The Tri-Reagent, thiazolyl blue tetrazolium bromide (MTT) and any other reagent was from Sigma Aldrich (Milan, Italy). For the contaminant both Glyphosate with its reactive AMPA and Glyphosate and AMPA separately were purchased from Biorad Laboratories (Milan, Italy).

3.3. Haemocytes preparation

Fresh pooled haemolymph was plated in 12-well plates (MXR transport activity; 1 mL/well) or 96-well plates (mRNA expression analysis; 300 µL/well) and left to settle for 30 minutes in the dark at 16°C, for cells to attach to the bottom. The medium was then removed and 200 µL of ASW was added, as well as the tested chemicals at the selected concentrations. Organization of the samples in the plates can be seen in figures 7 and 8.

MXR Assay

1 2 3 4 5 6 7 8 9 10 11 12

A B C D E F G H

Control

Glyphosate+ AMPA0.1 µg/L

Glyphosate+ AMPA-1µg/L

Glyphosate -0.1 µg/L

Glyphosate- 1 µg/L

AMPA-0.1 µg/L

AMPA-1 µg/L

Figure 7. Organization of the plates for the MXR transport activity analysis.

Gene Transcription

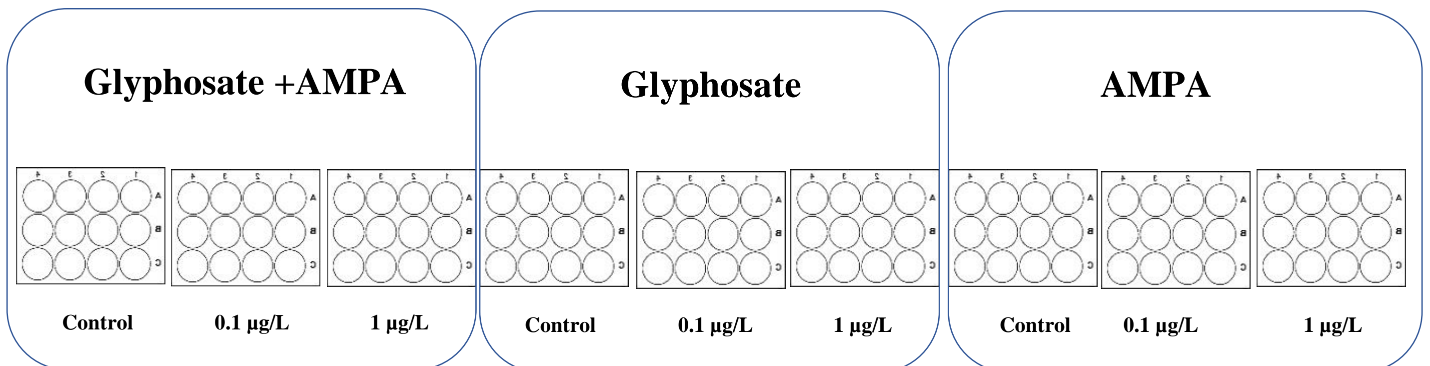

Figure 8. Organization of the plates for the mRNA expression analysis.

Treatments to evaluate basal relative activities and expression of P-gp transporters

For the evaluation of relative P-gp transport activity, haemocytes were exposed with the contaminant glyphosate and its metabolite AMPA, in the first trial together and in the second and third each separately. The lysis buffer Tricol was added for RNA isolation, 1mL for each two sample, this can be seen in figure 9. This combination of samples left the remaining of three treatments per concentration, that were renamed.

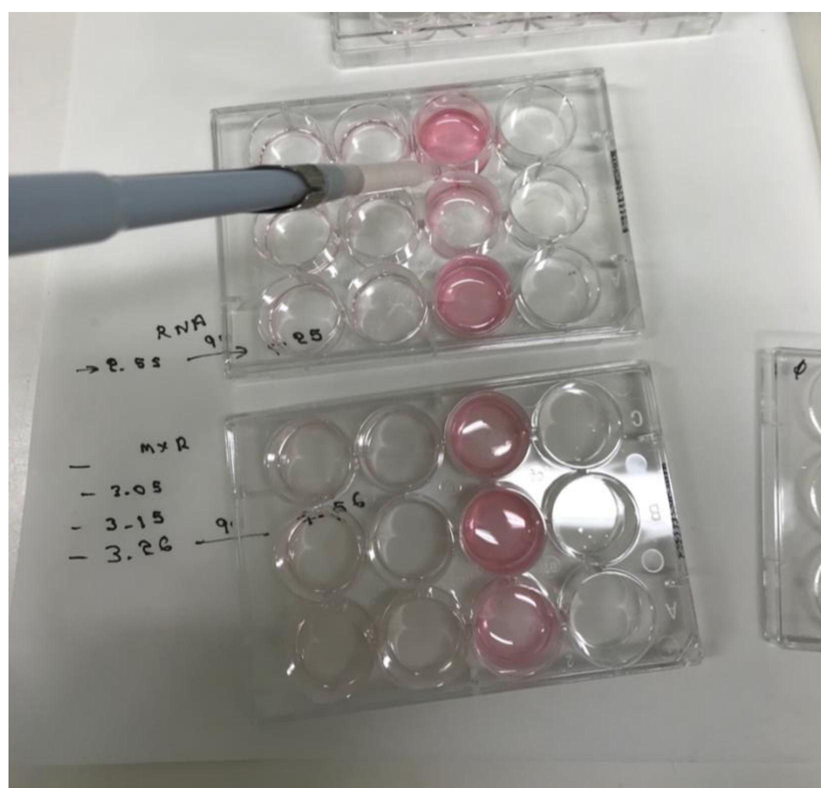

Figure 9. Plates with samples and Tricol for the mRNA expression analysis.

Treatments to investigate the effects of Glyphosate and AMPA on MXR transport activity

Following the haemocytes preparation, after the contaminant was added to the 12-well plates, it was left to settle 10 minutes in the dark. The contaminant for the first experimental trail had the combination of glyphosate and AMPA, the second had glyphosate and the third AMPA separately. At the end of the treatments, the medium was removed from control and treated samples, and replaced by 1 mL ASW containing 2.5 μM Rho 123. Cells were incubated for 90 min at 16 °C in the dark for the further processed for MXR activity assay.

3.4. MXR transport activity assay

The assay was developed using Rho123, a fluorescent model substrate for P-gp transporters, to examine cell-based transport (Smital et al., 2000). Rho123 shows a low to moderate rate of passive membrane permeation, allowing ABC efflux pumps to effectively extrude it (Luckenbach, Fischer and Sturm, 2014). As a result, the amount of Rho123 in the cell is an indicator of transporter activity: a weaker Rho123 fluorescence signal indicates higher transporter activity, while a stronger fluorescence signal indicates lower transporter activity (Franzellitti et al., 2016).

As mentioned above, haemocytes were subjected to the selected treatment in the presence of 2.5 µM Rho 123. Haemocytes were lysed with a 0.1% Triton-X100 solution prepared in phosphate-buffered saline (PBS) (500 µL/well) at the end of the incubation cycle. The samples were conveniently diluted and fluorescence measurements were performed using a Jasco FP-6200 fluorometer ($\lambda_{excitation}$ = 485 nm; $\lambda_{emission}$ = 530 nm). Plates were light-protected with aluminium foils during the procedure to avoid potential loss of Rho123 fluorescence intensity due to direct exposure to light.

3.5. RNA extraction from haemocytes

The RNA extraction protocol form mussels mantle was used to extract and characterize the amount of mRNA available on the haemocytes samples. First step, was to add chloroform and centrifuge 12 600rpm, for 15 minutes at 4ºC. This caused the samples to separate in three layers, the transparent supernatant, containing RNA, was transferred to 1.5 mL appendages.

Isopropanol (100%) was added to the appendages and left 10 minutes for incubation at room temperature. When finished the sample was centrifuge at 12 600rpm, for 10 minutes at 4ºC, the liquid part was then discarded, using a micropipette carefully to avoid touching the pellet. Then the sample was incubated on a thermal block at 50ºC, until the pallet was dry, followed by the addition of distilled water and vortex of the sample for resuspension. Furthermore, it was kept again on a thermal block one minute, to facilitate resuspension. The next step was to add LiCl 3M and vortex, then it was kept in the ice for 30 minutes. After, it was centrifuge at 13 500rpm, for 20 minutes at 4ºC; when finished the liquid was removed, 70% ethanol was added and then the sample was vortexed. A final centrifuge was done at 9 600rpm, for 6 minutes at 4ºC, continued by the removal of ethanol and the trying of the pellet using the thermal block. At the end diethyl pyrocarbonate (DEFC) was added and resuspension was achieved by vortexing and incubating in the thermal block for 1 minute.

CDNA preparation

Total RNA concentration and quality were verified, from the samples of RNA extracted from the haemocytes, through spectrophotometric analysis, that assessed the UV absorbance spectra of the samples (λ= 200 to 340 nm). The absorbance values (A) at 260 and 280 nm were used to calculate RNA concentration (A260), the ratio A260/ A280 addressing the occurrence of protein contaminations (cut-off values > 1.8 and < 2.0). Continuing, the first strand cDNA for each haemocyte sample was synthesized from 1 µg of total RNA using the iScript supermix following the manufacturer's protocol (Biorad Laboratories, Milan, Italy).

3.6. Selection of best-performing reference gene for qPCR analyses

Normalization is an important part of a successful qPCR assay because it compensates for sample-to-sample differences, allowing for comparisons of mRNA concentrations under various experimental conditions (Franzellitti *et al.*, 2015). Expression profiles of 6 established candidate reference transcript namely Actin (ACT), Tubulin (TUB), Helicase (HEL), Elongation factor 1 (EF1), 18s ribosomal RNA (18s rRNA) and 28s ribosomal RNA (28s rRNA) were analysed to identify best performing reference transcripts to achieve robust normalization of qPCR data in mussels haemocytes. qPCR reactions were performed in a final volume of 10 µL containing iTAq Universal Master Mix with ROX (BioRad Laboratories, Milan, Italy), diluted cDNA and primer pairs of ACT, TUB, HEL, EF1, 18s rRNA and 28s rRNA. A list of this reference genes with their acronym, primers, amplicon size, amplification efficiency, accession number and references can be seen in table 1.

Table 1. Acronym, primers, amplicon size, amplification efficiency, accession number and references for best-performing reference gene for qPCR analyses.

Acronym	Transcript name	Primers (5'-3')	Amplicon size (bp)	Amplification efficiency (%)	Accession number	References
Reference genes (assessed in haemocytes)						

18S	*18S rRNA*	TCGATGGTACGTGATATGCC CGTTTCTCATGCTCCCTCTC	90	95	L33451	(Dondero *et al.*, 2005)
28S	*28S rRNA*	AGCCACTGCTTGCAGTTCTC ACTCGCGCACATGTTAGACTC	142	94	DQ158078	(Ciocan *et al.*, 2011)
ACT	*Actin*	GTGTGATGTCATATCCGTAAGGA GCTTGGAGCAAGTGCTGTGA	120	114	AF157491	(Banni *et al.*, 2011)
TUB	*Tubulin*	TTGCAACCATCAAGACCAAG TGCAGACGGCTCTCTGT	135	102	HM537081	(Cubero-Leon *et al.*, 2012)
EF1	*Elongation factor-1α*	CGTTTTGCTGTCCGAGACATG CCACGCCTCACATCATTTCTTG	135	99	AB162021	(Ciocan *et al.*, 2011)
HEL	*Helicase*	GCACTCATCAGAAGAAGGTGGC GCTCTCACTTGTGAAGGGTGAC	129	132	DQ158075	(Cubero-Leon *et al.*, 2012)

Amplifications were carried out in a StepOne real-time qPCR system apparatus (Life Technologies), using a standard "fast mode" thermal protocol.

Critical Threshold (CT) values of each amplified transcript were imported to the "RefFinder" web-based tool to account for a comprehensive ranking calculated from the BestKeeper, geNorm and NormFinder along with comparative delta-CT method to calculate the transcript stability and identify the best performing candidate reference gene products.

According to RefFinder web based tool recommended comprehensive ranking, it shows that HEL and TUB reference gene transcript were more stable in expression than other candidate transcript.

In addition, according to other algorithms comparison, results indicate that ACT is stable in Delta CT (Figure 10), whereas 28S is stable in Normfinder (Figure 11). Furthermore, the three other methods, geNorm, BestKeeper and comprehensive gene stability method indicate that TUB and HEL are the most stable genes (Figure 12, 13 and 14). Therefore, based on stability analysis of candidate reference gene product, TUB was selected as the best performing reference gene product, and TUB/HEL pair was selected as the best performing combination reference gene product to be used for normalization of the real time qPCR data in *M. galloprovincialis* haemocytes.

3.7. Quantitative real-time PCR analysis of ABCB mRNA expressions

For absolute quantification of the ABCB transcripts performed in haemocytes, qPCR standards for each target transcript were prepared by serial dilution of the linearized plasmid DNAs containing the specific transcripts to obtain a standard curve of threshold cycle (CT) values vs the logarithmic DNA amount.

A normalization factor was determined using the geNorm software for the relative expression analyses of the target transcripts (Vandesompele *et al.*, 2002) and was used for precise normalization of qPCR data based on the expression levels of the best performing reference transcripts in the analysed samples. (Franzellitti and Fabbri, 2013b; Franzellitti *et al.*, 2013). The most stable reference gene products under the different experimental conditions tested were TUB and HEL. A comparative CT approach was used to measure the relative expression of target mRNAs in relation to reference gene products (Schmittgen and Livak, 2008) using the StepOne software tool (Life Technologies, Milan, Italy). Data were reported as mean ± SEM of the normalized relative expression (log2-normalized fold changes) with respect to control samples.

The immune response genes and lysosomal genes analysed in the real-time PCR, with their acronym, primers, amplicon size, amplification efficiency, accession number and references can be seen in the table 2.

Table 2. Immune response genes and lysosomal genes analysed in the real-time PCR with their acronym, primers, amplicon size, amplification efficiency, accession number and references.

Acronym	**Transcript name**	**Primers (5'-3')**	**Amplicon size (bp)**	**Amplification efficiency (%)**	**Accession number**	**References**
Target genes (assessed in haemocytes)						
ABCB	*P-glycoprotein*	CACCATAGCCGAGAACATCC CTCCACGCTCTCCAACTAG	139	112	EF057747	(Franzellitti and Fabbri, 2013a)
lys	Lysozyme	ATGTGGAATCTGAAGGACTTGT CCAGTATCCAATGGTGTTAGGG	368	124	AF334665	(Balbi *et al.*, 2017))
MYTL b	mytilin b	TGAAGGCAGGAGTTATTCTGGC ACAACGAAGACATTTGCAGTAGC	100	105	AF162336	(Pallavicini et al., 2008)
MYTC	myticin c	ATTTGCTACTGCCTTCATTG TCCATCTCGTTGTTCTTGTC	100	97	AF162334	(Pallavicini et al., 2008)

ctsl	Cathepsin L	CCGAGGCTTCATACCC ATATAC CGACAGCGGACATCAA ATCT	129	92	AY618311	(Capolupo *et al.*, 2018)
hex	Hexosaminidase	GATACTCCAGGACACA CTCAATC CTGGTCCATAGCTACC ATCAAATA	97	101	EU339934	(Capolupo *et al.*, 2018)
gusb	ß-Glucuronidase	GCGGTCATTATCTGGT CTGTAG CCGGTCTTGTTGGGTC TAAAT	112	120	EU339935	(Capolupo *et al.*, 2018)

3.8. Statistical analysis

Data on qPCR quantification of ABCB copy numbers in haemocytes and rhodamine efflux assay data were analysed using non-parametric Mann-Whitney U-test. These statistical analyses were performed using SigmaPlot ver.13 (Systat Sostware). Data from relative qPCR analysed were evaluated with the REST software (Vandesompele *et al.*, 2002) that uses a randomisation test with a pair- wise reallocation to assess the statistical significance of the differences in expression between each treatment-exposed group and the controls. In all approaches, $p < 0.05$ was set as the threshold level of statistical significance.

4. Results

4.1. Analysis of the most stable reference gene

Normalization of the qPCR was achieved by the analysis of the most stable reference genes. The analysis of the results of each gene was performed in the web Refinder, which uses different methods to analyse from most stable to least stable genes.

The graph obtained for the Delta CT method showed that ACT and 28S were the most stable genes (Figure 10).

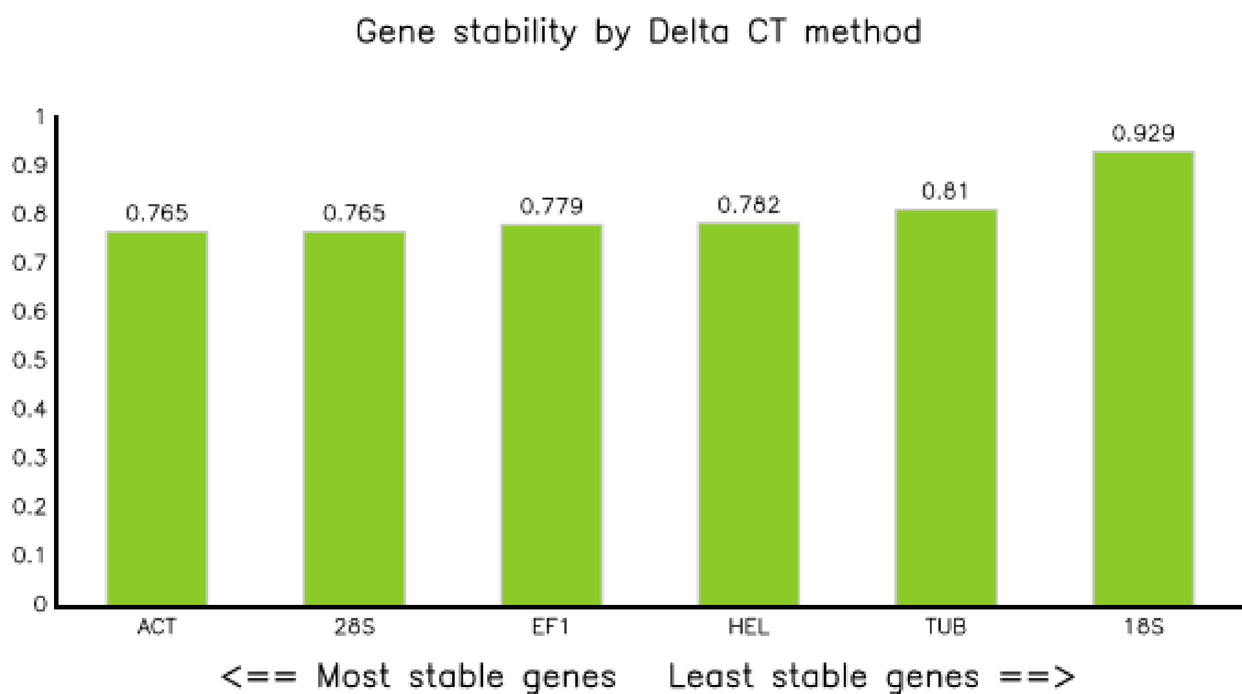

Figure 10. PCR results of the gene stability analysis of Actin (ACT), Tubulin (TUB), Helicase (HEL), Elongation factor 1 (EF1), 18s ribosomal RNA (18s rRNA) and 28s ribosomal RNA (28s rRNA), for the Delta CT method.

Similar results were obtained with the gene stability normFinder being 28S the most stable followed by ACT (Figure 11).

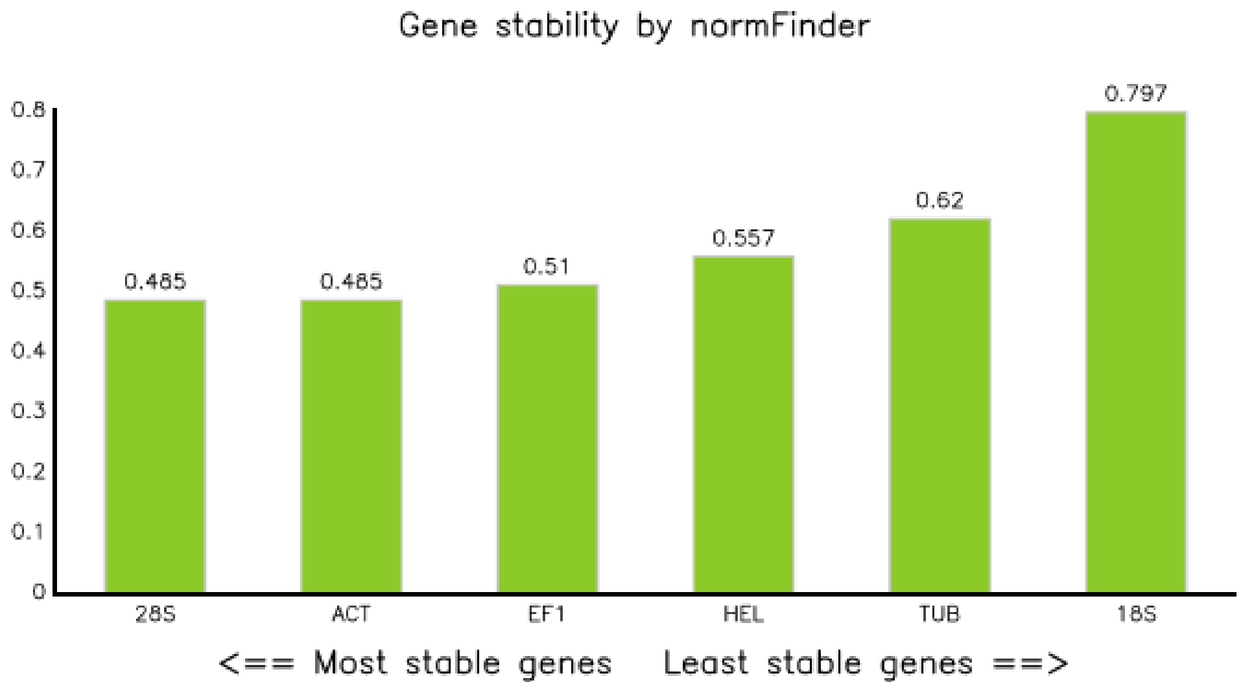

Figure 11. PCR results of the gene stability analysis of Actin (ACT), Tubulin (TUB), Helicase (HEL), Elongation factor 1 (EF1), 18s ribosomal RNA (18s rRNA) and 28s ribosomal RNA (28s rRNA), for the NormFinder method.

In comparison, both TUB and HEL, were obtained as most stable genes with the method Genorm, this can be seen in figure 12.

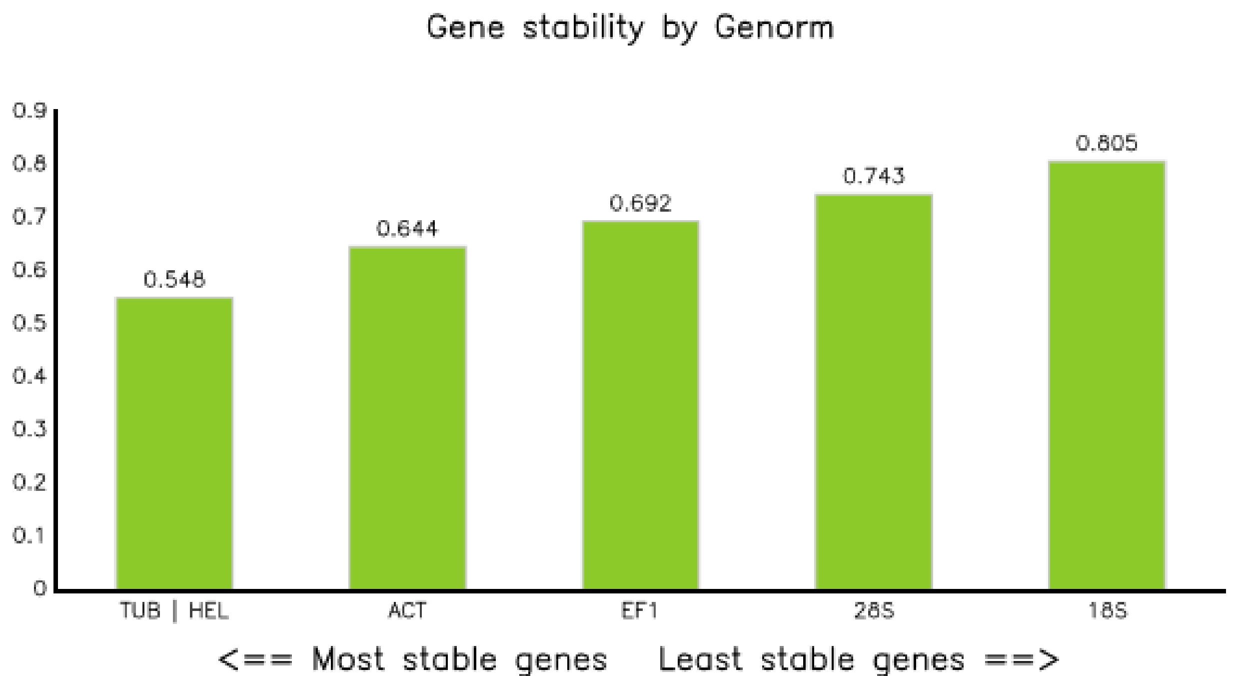

Figure 12. PCR results of the gene stability analysis of Actin (ACT), Tubulin (TUB), Helicase (HEL), Elongation factor 1 (EF1), 18s ribosomal RNA (18s rRNA) and 28s ribosomal RNA (28s rRNA), for the Genorm method.

Likewise, for the BestKeeper method both TUB and HEL appeared to be the most stable genes, figure 13.

Figure 13. PCR results of the gene stability analysis of Actin (ACT), Tubulin (TUB), Helicase (HEL), Elongation factor 1 (EF1), 18s ribosomal RNA (18s rRNA) and 28s ribosomal RNA (28s rRNA), for the BestKeeper method.

As can be seen in the figure 14, in the comprehensive gene stability method, the most stable gene is TUB followed by HEL.

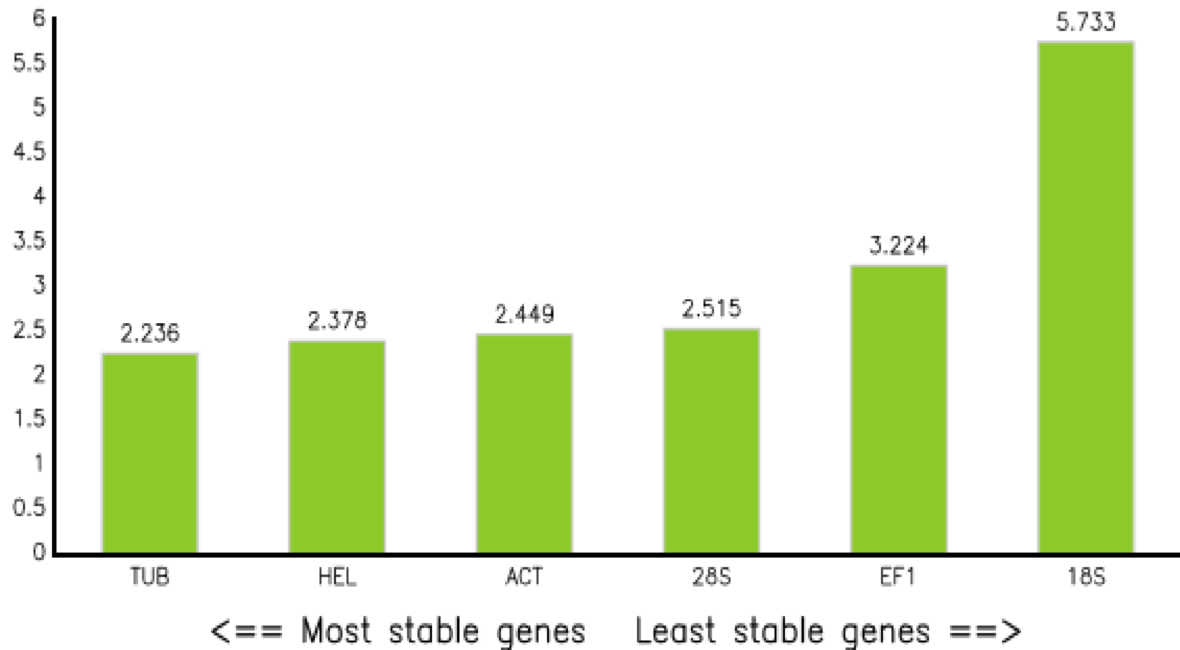

Figure 14. PCR results of the gene stability analysis of Actin (ACT), Tubulin (TUB), Helicase (HEL), Elongation factor 1 (EF1), 18s ribosomal RNA (18s rRNA) and 28s ribosomal RNA (28s rRNA), for the comprehensive gene stability method.

Therefore, as three of the five methods concluded that TUB was the most stable gene followed by HEL, those two genes where chosen for the normalization of the qPCR.

4.2. Effect of glyphosate and AMPA on MXR activity and ABCB mRNA expression

4.2.1. Effects of combination of Glyphosate and AMPA on MXR efflux activity and ABCB mRNA expression

In mussel haemocytes, changes in MXR transport activity were investigated by measuring cell accumulation of the model fluorescent substrate Rho123 (Smital *et al.*, 2000) in the presence of glyphosate and AMPA.

Mussel haemocytes were maintained in the presence of 0.1 and 1 µg/L combination of glyphosate and AMPA. Compared to controls, Rho 123 accumulation was significantly (*P < 0.05*) increased by both treatments in the haemocytes, indicating reduced MXR activities (Figure 15 A). In 0.1 µg/L treatment, levels of the *ABCB* transcripts (encoding P-gp) were unchanged (Figure 15 B), while significant down-regulation (*0.05>P*) of the *ABCB* transcript was observed at 1 µg/L (Figure 15 B).

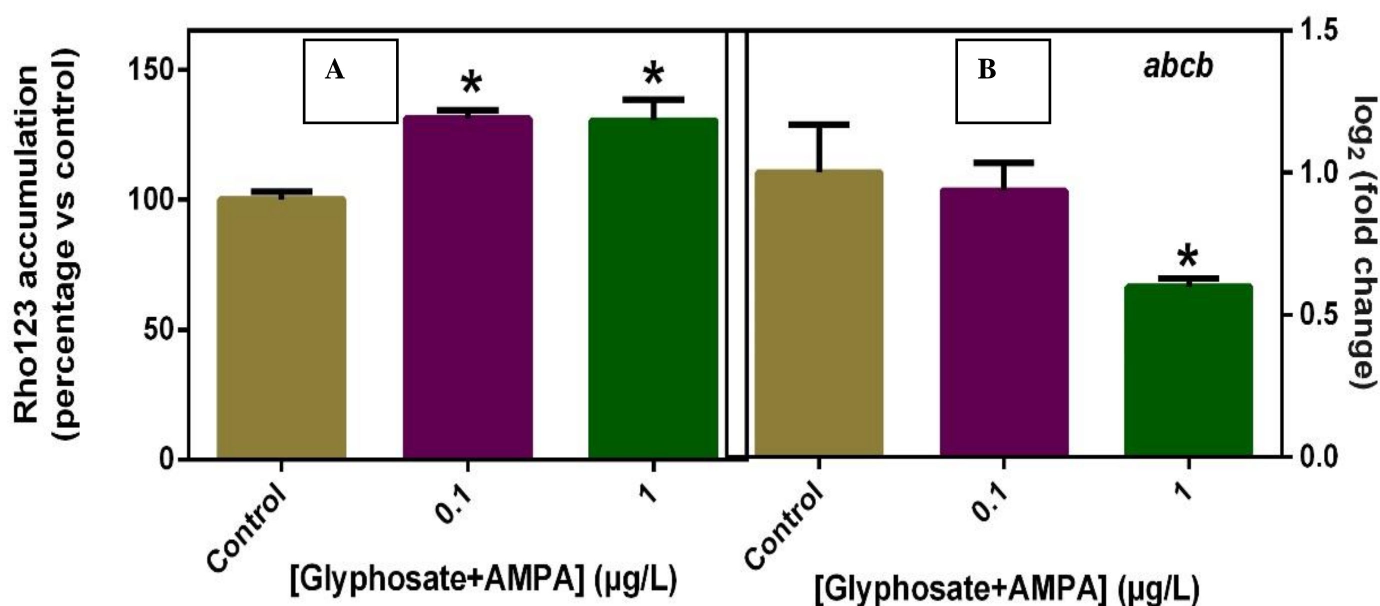

Figure 15. Effects of 0.1 and 1 µg/L combination of glyphosate and AMPA on the MXR system in haemocytes of adult Mediterranean mussels. (A) Rho123 accumulation in haemocytes. Data are expressed as mean ± SEM of 3 experiments carried out in 12- well plates (3 replicate wells for each sample) (N=3) of the variation vs respective controls (ctr; untreated haemocytes). (B) Levels of ABCB (encoding the P-glycoprotein, P-gp) expressions in haemocytes treated with combination of glyphosate and AMPA at the nominal concentrations of 0.1 and 1 µg/L. Values (mean ± SEM) are expressed as the relative variations (normalized fold changes) between each treatment and control samples (N=3). *P < 0.05 vs control.

4.2.2. Effects of Glyphosate on MXR efflux activity and ABCB mRNA expression

In this trail, mussel haemocytes were maintained in the presence of 0.1 and 1 µg/L of glyphosate. Compared to controls, Rho 123 accumulation was significantly (*P < 0.05*) decreased by both treatments in the haemocytes (Figure 16 A). In 0.1 µg/L treatment, levels of the *ABCB* transcripts significantly decreased (Figure 16 B), while an increase of the *ABCB* transcript was observed at 1 µg/L (Figure 16 B).

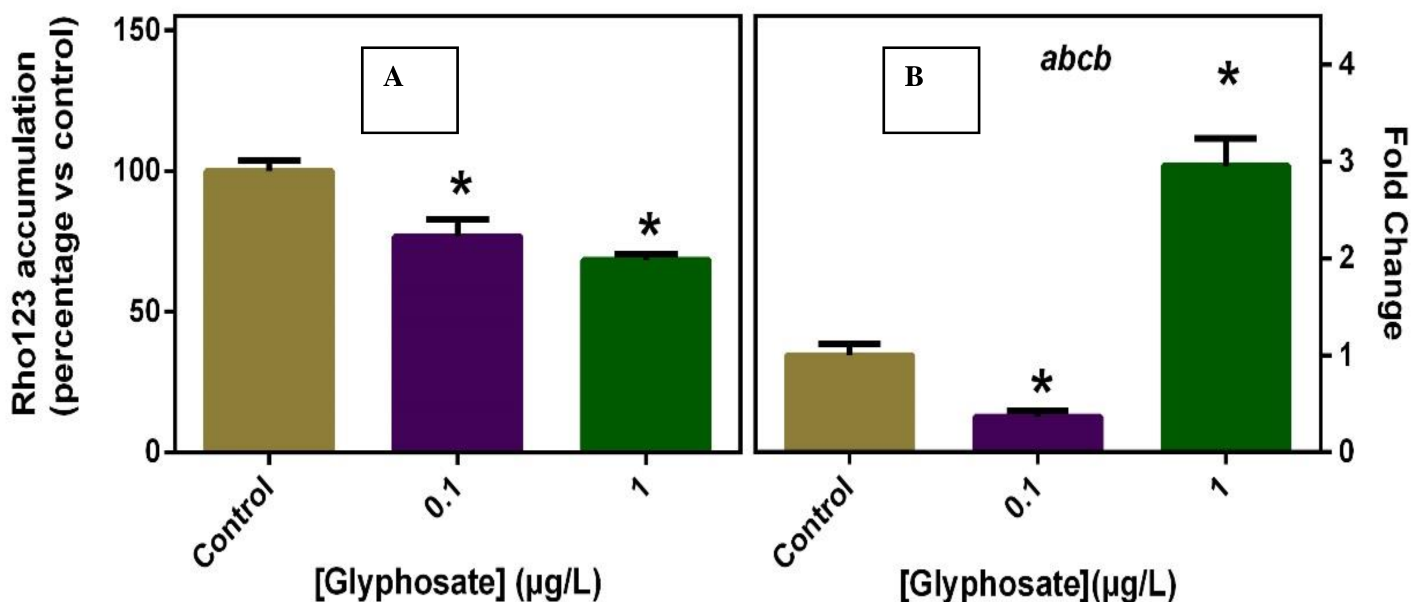

Figure 16. Effects of 0.1 and 1 µg/L glyphosate on the MXR system in haemocytes of adult Mediterranean mussels. (A) Rho123 accumulation in haemocytes. Data are expressed as mean ± SEM of 3 experiments carried out in 12- well plates (3 replicate wells for each sample) (N=3) of the variation vs respective controls (ctr; untreated haemocytes). (B) Levels of ABCB (encoding the P-glycoprotein, P-gp) expressions in haemocytes treated with combination of glyphosate and AMPA at the nominal concentrations of 0.1 and 1 µg/L. Values (mean ± SEM) are expressed as the relative variations (normalized fold changes) between each treatment and control samples (N=3). *$P < 0.05$ vs control.

4.2.3. Effects of AMPA on MXR efflux activity and ABCB mRNA expression

Moreover, mussel haemocytes were maintained in the presence of 0.1 and 1 µg/L of AMPA. Compared to controls, Rho 123 accumulation was significantly (*P < 0.05*) decreased by both treatments in the haemocytes (Figure 17A). For both, 0.1 µg/L and 1 µg/L treatment, levels of the *ABCB* transcripts were unchanged (Figure 17B).

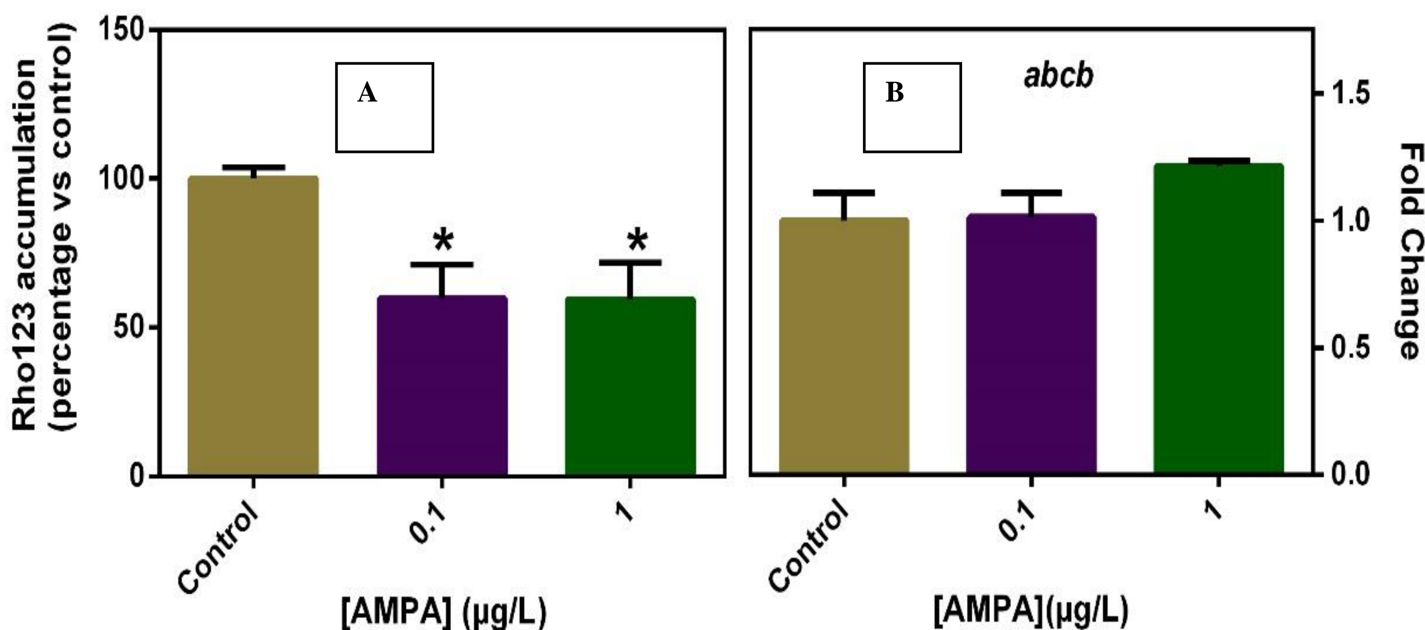

Figure 17. Effects of 0.1 and 1 µg/L of AMPA on the MXR system in haemocytes of adult Mediterranean mussels. (A) Rho123 accumulation in haemocytes. Data are expressed as mean ± SEM of 3 experiments carried out in 12- well plates (3 replicate wells for each sample) (N=3) of the variation vs respective controls (ctr; untreated haemocytes). (B) Levels of ABCB (encoding the P-glycoprotein, P-gp) expressions in haemocytes treated with combination of glyphosate and AMPA at the nominal concentrations of 0.1 and 1 µg/L. Values (mean ± SEM) are expressed as the relative variations (normalized fold changes) between each treatment and control samples (N=3). *P < 0.05 vs control.

4.3. Effects of glyphosate and AMPA on gene transcription

4.3.1. Effects of combination of glyphosate and AMPA on gene transcription

The effects of haemocytes exposure to combination of glyphosate and AMPA (0.1 or 1 µg/L) on transcription of 6 genes involved in different physiological functions; immune responses (genes encoding lysozyme, lys; mytilin b, mytlB; myticin c, mytcC), lysosomal system (genes encoding cathepsin L, ctsl; ß-glucuronidase, gusb; hexosaminidase, hex) were evaluated. All transcripts involved in the immune response (lys, mytlB, mytcC) showed no significant variations following treatments except significant up regulation of mytlB at 0.1 µg/L. The both ctsl and hex transcripts were significantly up regulated at 0.1 µg/L combination of glyphosate and AMPA. A significant down-regulation was observed for all transcripts encoding lysosomal enzymes at 1 µg/L concentrations (Figure 18).

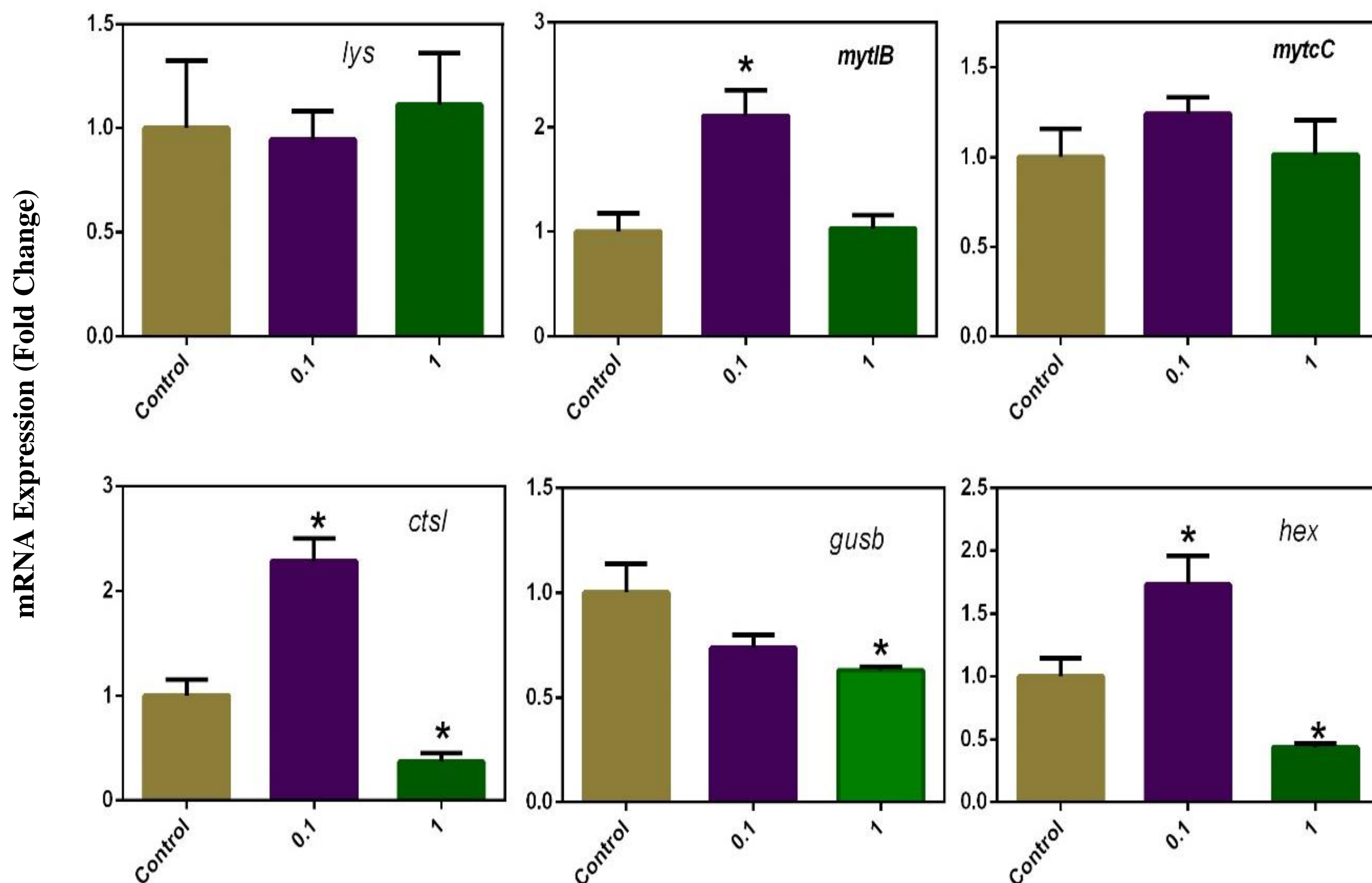

Figure 18. Effects of combination of glyphosate and AMPA (0.1 and 10 µg/L) on gene trancription in *M. galloprovincialis* haemocytes. Relative expression of transcripts involved in (A) immune responses (genes encoding lysozyme, lys; mytilin b, MYTLb; myticin c, mytcC), (C) lysosomal system (genes encoding cathepsin L, ctsl; ß-glucuronidase, gusb; hexosaminidase, hex). Data are reported as mean ± SEM of 3 experiments carried out in 12-multiwell plates (3 replicate wells for each sample) (N=4). Values represent the relative variation (normalized fold changes) vs controls (untreated haemocytes) (*p < 0.05).

4.3.2. Effects of glyphosate on gene transcription

When haemocytes where exposed to glyphosate (0.1 or 1 µg/L), some of the effects on transcription of 6 genes involved in different physiological functions; immune responses, lysosomal system were evaluated. The transcripts involved in the immune response (lys) showed a significant decrease variations following treatments in both concentrations. MytB showed no significant variation in ether of the concentrations, on the other hand, MytlC had a significant increase in both 0.1 µg/L and 1 µg/L concentrations. All transcripts involved in the lysosomal system (ctsl, gusb and hex) showed a significant

down regulation following treatments except no significant variation of ctsl at 1 μg/L (Figure 19).

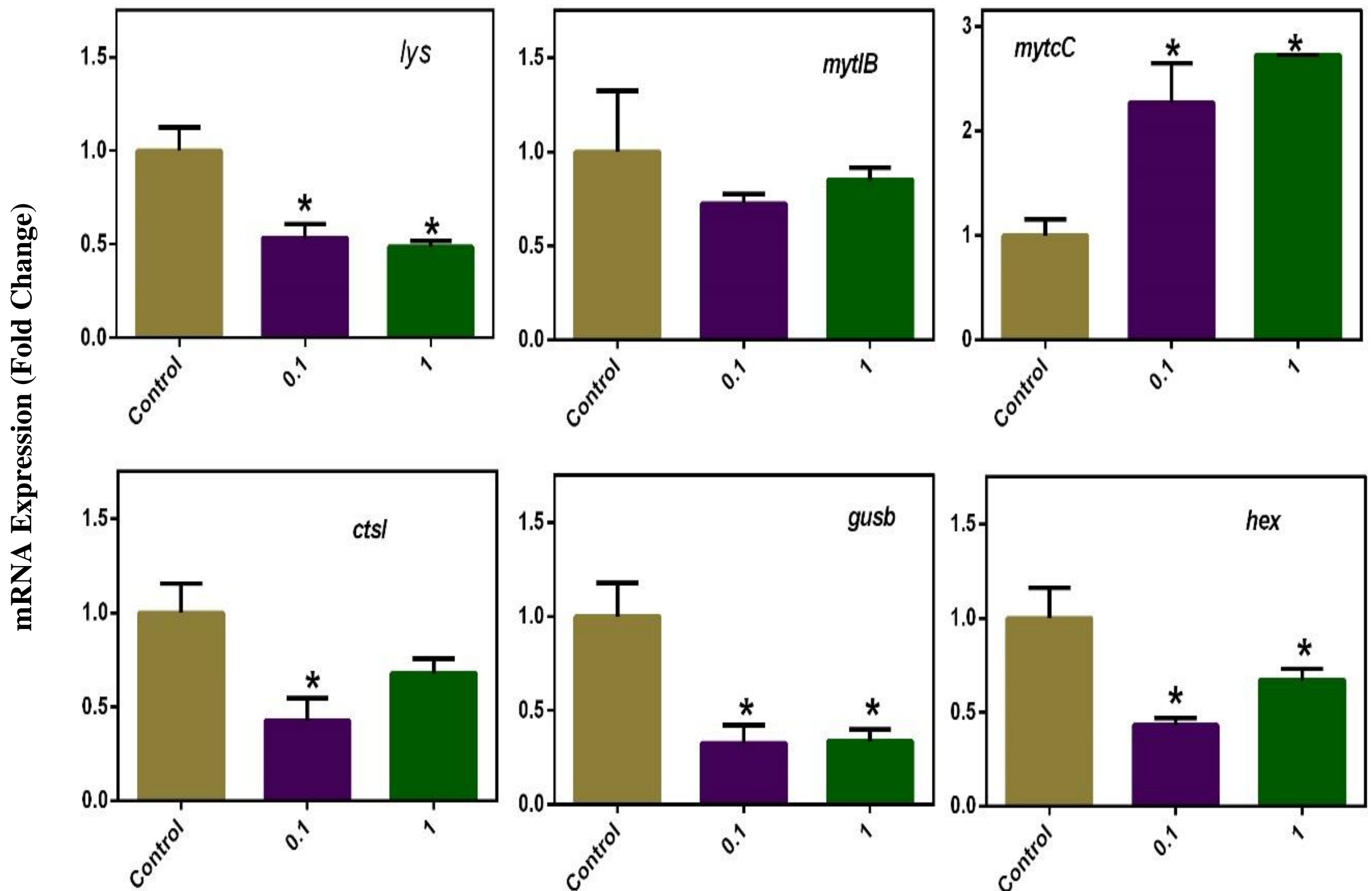

Figure 19. Effects of glyphosate (0.1 and 10 μg/L) on gene trancription in *M. galloprovincialis* haemocytes. Relative expression of transcripts involved in (A) immune responses (genes encoding lysozyme, lys; mytilin b, MYTLb; myticin c, mytcC), (C) lysosomal system (genes encoding cathepsin L, ctsl; ß-glucuronidase, gusb; hexosaminidase, hex). Data are reported as mean ± SEM of 3 experiments carried out in 12-multiwell plates (3 replicate wells for each sample) (N=4). Values represent the relative variation (normalized fold changes) vs controls (untreated haemocytes) (*p < 0.05).

4.3.3. Effects of AMPA on gene transcription

For the haemocytes exposed to AMPA (0.1 or 1 μg/L), some of the effects on transcription of 6 genes involved in different physiological functions; immune responses, lysosomal system were evaluated. The transcripts involved in the immune response (lys) showed a significant decrease variations at 0.1 μg/L concentration. MytB had a

significant decrease variations at 1 µg/L concentration, on the other hand, MytlC had a significant increase at 0.1 µg/L and a decrease at 1 µg/L concentrations. All transcripts involved in the lysosomal system (ctsl, gusb and hex) showed a significant down regulation following treatments except for 0.1 µg/L ctsl that had a significant up regulation and a no significant variation of hex at 0.1 µg/L (Figure 20).

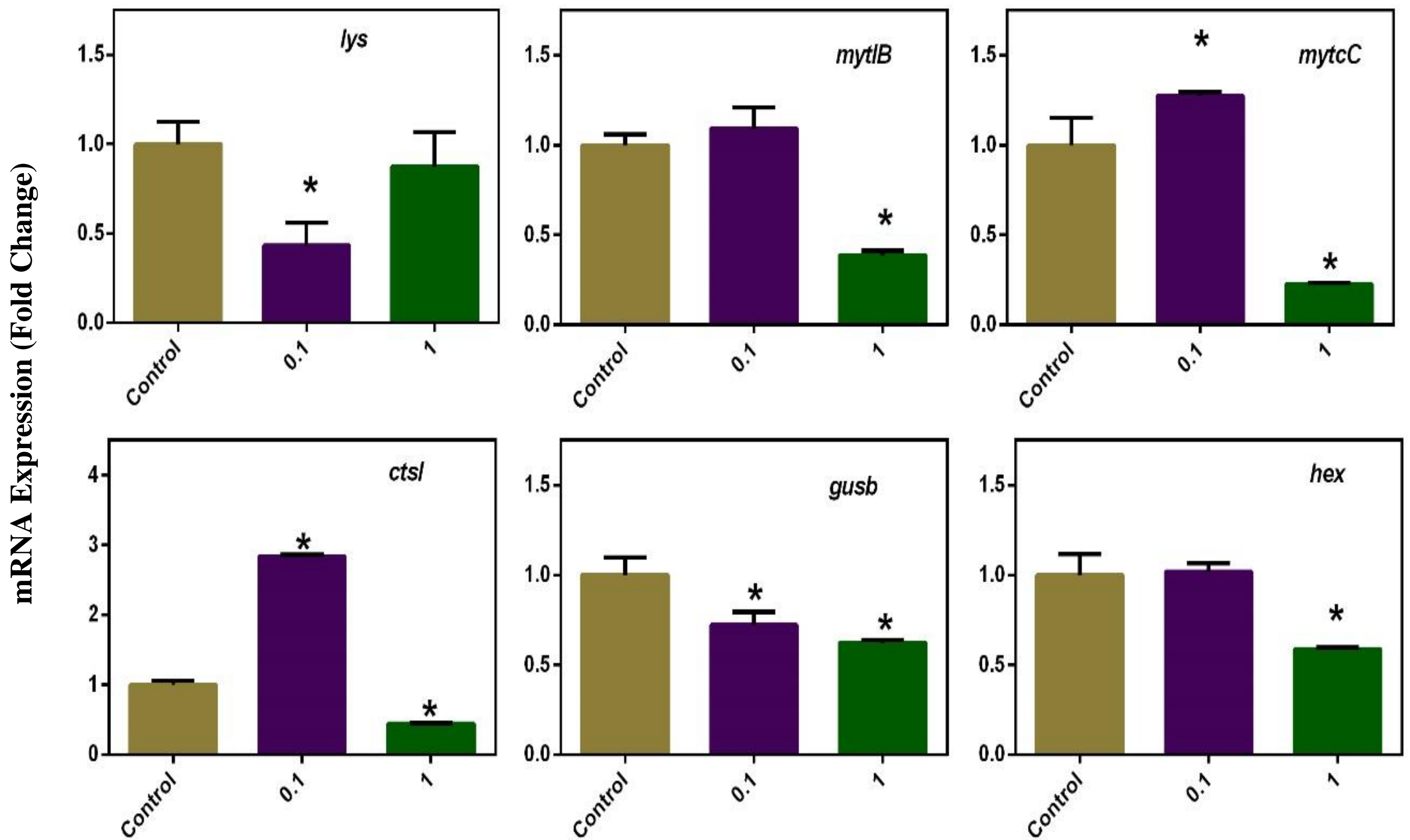

Figure 20. Effects of AMPA (0.1 and 10 µg/L) on gene trancription in *M. galloprovincialis* haemocytes. Relative expression of transcripts involved in (A) immune responses (genes encoding lysozyme, lys; mytilin b, MYTLb; myticin c, mytcC), (C) lysosomal system (genes encoding cathepsin L, ctsl; ß-glucuronidase, gusb; hexosaminidase, hex). Data are reported as mean ± SEM of 3 experiments carried out in 12-multiwell plates (3 replicate wells for each sample) (N=4). Values represent the relative variation (normalized fold changes) vs controls (untreated haemocytes) (*$p < 0.05$).

5. Discussion

The findings of this work aim to add to our understanding of the regulatory processes that lead to haemocytes physiological chemoresistance, which is a significant benefit in improving mussel adaptability to contaminants in their environments (Bard, 2000). To this end, investigations were performed on the MXR transport activity, expression of an ABCB (encoding P-gp) transcript, as well as effects on gene transcripts in mussels treated with chemicals that interact with adrenergic and serotonergic signaling. Aiming to link the transcription-level changes that have already been discovered (Franzellitti and Fabbri, 2013b) to possible functional deficiencies that contribute to negative haemocyte physiology outcomes.

The Multixenobiotic Resistance (MXR) system is a broad-spectrum defensive mechanism made up of membrane and intracellular active transporters that operate as a first line of defense against environmental toxins, preventing their buildup and damaging effects (Bielen *et al.*, 2016). The results of this investigation demonstrated that samples treated with both glyphosate and AMPA accumulated more of the model fluorescent substrate Rho123, implying a reduction in MXR overall efflux activity. This effect appears not linked to the transcriptional regulation of the genes encoding one of the main MXR-related ABC transporters P-gp (ABCB) when the concentration was lower and a significant reduction could be seen for the higher concentration, the same effects where observed when exposed to Styrene (Wathsala *et al.*, 2018).

Certainly, ABCB was slightly affected, this finding may lead to hypothesis that glyphosate and AMPA might cause more significant alterations on ABCC compared to controls. Which would indicate that this contaminant acts as a MXR substrate causing Rho123 accumulation, without related transcriptional modulation.

On the other hand, both samples exposed to glyphosate and AMPA separately showed a decrease accumulation of Rho123, suggesting no alteration of the MXR efflux activity. This effect appears not linked to the transcriptional regulation of P-gp for samples exposed to AMPA, contrastingly glyphosate exposed samples showed significant variation of P-gp.

The physiological significance of this unusual ABCB/ABCC relative expression in haemocytes could be explained by their separate but partially overlapping roles as phase 0 (P-gp) or phase III (Mrp) transporters in biotransformation and detoxification processes (Bard, 2000; Leslie, Deeley and Cole, 2005; Luckenbach, Fischer and Sturm, 2014).

Given its low baseline mRNA expression and function as a phase 0 transporter, P-gp may be classified as a stress-inducible transporter with mostly defensive activities, and its activity was found to correlate well with pollution levels (Žaja *et al.*, 2006). Mrp can also be thought of as a "dual-functioning transporter" (Shipp and Hamdoun, 2012), serving both defensive and physiological purposes. The fact that the expression patterns of mussel ABCB but not ABCC were associated with those of stress-induced gene products such the stress-inducible metallothionein mt20 in prior research further points to a connection (Franzellitti *et al.*, 2010).

Because haemolymph flows through different tissues and organs to collect products involved on the cellular processes (Bardales, Cascallana and Villamarin, 2011), the amount of potential MXR substrates to which haemocytes are exposed may be determined by the cumulative detoxification activities of animal tissues rather than simply by the amount of pollution in the environment (Žaja *et al.*, 2006). We can postulate that haemocytes have developed a unique MXR system to support their role as the animal's internal defense, if the activities of P-gp and Mrp transporters are as stated above. Given its role as a scavenger of intermediates and byproducts from metabolic and oxidative reactions underlying haemocyte immune and stress response systems, Mrp may be the most important contributor to detoxification (Donaghy *et al.*, 2009). This has been proven to be the case in previous studies (Franzellitti *et al.*, 2016; Wathsala *et al.*, 2018). Maintaining a limited ABCB mRNA expression under physiological settings may be part of a preparative approach to thrive more successfully with environmental unpredictability, as has been indicated for other stress-induced proteins (Franzellitti and Fabbri, 2005). P-gp expression can be effectively induced in this hypothesis to keep MXR activity ongoing in stressful situations.

Changes in efflux activity are known to not necessarily correspond to transcriptional patterns of genes encoding ABC transporters. This is most likely owing to the complicated regulation of ABC transporter synthesis and activity, which includes transcriptional, post-transcriptional, and post-translational processes, as well as the complexities of substrate-transporter interactions (Scotto, 2003; Hennessy and Spiers, 2007). Recent research found that the pharmaceuticals propranolol, carbamazepine, and fluoxetine, all of which are potential substrates for MXR transporters, affected mussel ABCB and/or ABCC transcription, as well as changes in MXR efflux activity (Franzellitti *et al.*, 2016), implying that contaminants that act as substrates or inhibitors of ABC pumps may cause transcriptional changes. The increased need for P-

gp transporters that mediate glyphosate and AMPA extrusion may have triggered the observed positive regulation of ABCB transcription (Wathsala *et al.*, 2018).

To gain further insights into the possible mechanisms behind glyphosate effects in *M. galloprovincialis*, expression changes of further 6 transcripts were analyzed. These transcripts are involved in lysosomal responses and immune responses. Main effects were observed for both lysosomal and immune response in all treatments.

Lysosomes have been found in mussels as potential targets for glyphosate toxicity. In fact, earlier research has found a decrease in lysosomal membrane stability in haemocytes (Matozzo *et al.*, 2018). In accord, majority of the samples in our investigation exhibited a significant down-regulation of transcripts encoding lysosomal enzymes, indicating the beginnings of a dysfunctional lysosomal state. The lysosome enzymes - glucuronidase (GUSB) and hexosaminidase (HEX), in particular, are used as indicators to evaluate changes in lysosomal size and membrane stability (Marigómez *et al.*, 2005; Izagirre *et al.*, 2009). GUSB and HEX transcripts were shown to be down-regulated in mussel haemocytes, indicating a decrease in lysosomal membrane stability and loss of lysosomal functionality (Izagirre *et al.*, 2014). We may add that impairment of lysosomal functions has been linked to lower scope for growth in bivalves and animal health status, which could forecast effects at the population level, pending data on the function and dynamics of the lysosomal system across mussel development (Viarengo *et al.*, 2007).

Induction of an immune response, as that observed in this study by means of up regulation of transcripts encoding the main mussel antimicrobial peptides mytilin b (MYTLb) and myticin c (MYTCc), is a known effect of glyphosate, being observed in adults mussel exposed 21 days to 10, 100, and 1000 µg/L glyphosate (Milan *et al.*, 2018). Therefore, the up regulation of this genes, shown in this study, is a response to the microbial alterations that occur as a consequence of glyphosate exposure.

Similar results were obtained in previous research, for both lysosomal and immune response (Wathsala *et al.*, 2018), indicating that this respond mechanisms in mussels are commonly affected by different contaminants. On the whole, these results indicate the potential of glyphosate to impair haemocytes in Mediterranean mussel.

6. Conclusion

The molecular mechanisms of action of glyphosate in the marine bivalve *M. galloprovincialis* were investigated in this study, based on the results obtained the following conclusions can be made:

- The most stable reference gene was TUB and the TUB/HEL pair was chosen as the best performing combination reference gene for normalizing real-time qPCR data.
- The decreased MXR transport activity seen in this study when exposed to glyphosate contamination may have been caused by transcriptional control of ABCB (P-gp), suggesting that disruption of transduction pathways by external signals may have important implications on haemocyte function. P-gp is likely elevated under stress conditions, but it is hypothesized that Mrp, as a phase III transporter, provides a major support to physiological MXR function.
- As for the effects of this contaminant in the gene transcription, the lysosomal genes were shown to be down-regulated in mussel haemocytes, indicating a decrease in lysosomal membrane stability and loss of lysosomal functionality. Immune genes, on the other hand, had an up regulation, as a response to the microbial alterations that occur as a consequence of glyphosate exposure.

7. Future studies

This work has made it possible to know the effects of glyphosate on *M. galloprovincialis* haemocytes, considering data on MXR transport activity, expression of an ABCB (encoding P-gp) transcript, as well as effects on gene transcripts. However, some aspects, have been left out of the study. Therefore, the following future lines of research are proposed:

- Perform an analysis of the expression of the ABCC (encoding Mrp) transcript, with the same conditions and contaminant concentrations used in this study. To corroborate the hypothesis that Mrp may be the most important contributor to detoxification.
- Carry out the same study but using a higher concentration of contaminant (100µg/L) since the presence of glyphosate and AMPA is increasing in marine environments.
- Analyse more genes involved in other functions of the mussel, such as shell biogenesis or neuroendocrine signalling, to observe their transcriptional effect.

8. References

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