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MOLECULAR MECHANISMS CONTROLLING PHYSIOLOGICAL PLASTICITY IN MARINE MUSSELS UNDER THE INFLUENCE OF NATURAL AND ANTHROPOGENIC STRESS FACTORS

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Content

Abbreviations

5-HT1	type 1 serotonin receptor
BPA	bisphenol A
CA	carbonic anhydrase
CAT	catalase
CS	chitin synthase
CTSL	cathepsin L
EC50	toxicant concentrations causing 50% reduction on percentage of normal D-larvae
EP	extrapallial protein
GST	glutathione s-transferase
GUSB	ß-glucuronidase
HEX	hexosaminidase
LMS	lysosomal membrane stability
LYS	lysozyme
MeER1	type 1 estrogen receptor
MeER2	type 2 estrogen receptor
MPs	microplastics
Mrp	multidrug resistance related protein
MT10	metallothionein 10
MT20	metallothionein 20
MXR	multixenobiotic resistance
MYTCc	myticin c
MYTLb	mytilin b
Pgp	P-glycoprotein
PS-MPs	polystyrene MPs
qPCR	quantitative real time PCR
SCFAs	short-chain fatty acids
SOD	superoxide dismutase

Abstract

Marine mussels are exceptionally well-adapted to live in transitional habitats where they are exposed to fluctuating environmental parameters and elevated levels of natural and anthropogenic stressors throughout their lifecycle. However, there is a dearth of information about the molecular mechanisms that assist in dealing with environmental changes. This project aims to investigate the molecular mechanisms governing acclimatory and stress responses of the Mediterranean mussel (*Mytilus galloprovincialis)* by addressing relevant life stages and environmental stressors of emerging concern. The experimental approach consisted of two phases to explore (i) the physiological processes at early life history and the consequences of plastic pollution and (ii) the adult physiology processes under natural habitats.

As the first phase of the study, I employed a plastic leachate (styrene monomer) and polystyrene microplastics to understand the modulation of cytoprotective mechanisms during the early embryo stages. Results revealed the onset of transcriptional impairments of genes involved in MXR-related transporters and other physiological processes induced by styrene and PS-MPs, indicating sub-lethal impacts that could increase the larvae and adult vulnerability toward further environmental stressors.

In the second phase, I focused on adult life stages of mussels. As a preliminary analysis, microbiota profile of mussels at the tissue scale and its surrounding water was explored to understand distinctive microbiota structures that may reflect peculiar adaptations to the respective tissue functions. As part of the ongoing investigation of mussel transcriptional changes in the natural setting, I investigated the stability of common reference genes in the digestive gland of adult mussels representing different conditions such as location, season, and gender. In the present work, we validated two reference genes, 18S and 28S, for qPCR analysis of mussels and, based on the results obtained from four programs, to be most

suitable as reference genes. The broader experiment has been implemented to understand the variability of transcriptional profiles in the mussel digestive glands in the natural setting. All the genes employed in this study have shown possibilities to use as molecular biomarker responses throughout the year for monitoring the physiology of mussels living in a particular environment and, in turn, more properly detecting changes in the environment. As a whole, my studies provide insights into the interactions between environmental parameters, especially seawater parameters, and intrinsic characters (gender, reproductive stages), and physiology of marine bivalves, and it could help to interpretation of responses correctly under stress conditions and climate change scenarios.

General Introduction

The occurrences of pollutants and changing of oceanic parameters have been observed in marine waters in recent decades (González-De Zayas et al., 2020; Mearns et al., 2019; S. Ben Haj, 2010; Villarrubia-Gómez et al., 2018). However, the major concern is the extent of animal capacity to cope with this rapid changes (Evans and Hofmann, 2012). Marine mussels (*Mytilus* spp.) surpass deprived environmental factors and adapted to live in harsh environments characterized by a wide array of abiotic and biotic parameters, which makes them ideal model organisms for studying physiological alterations driven by cumulative actions of global warming and chemical pollution (Franzellitti et al., 2010; Viarengo et al., 2007). Scientific evidence outlines changes in mussel physiological functions that have been linked to both anthropic and natural stressors (Franzellitti et al., 2020; Fernández et al., 2010; Petes et al., 2008). However, there is a dearth of information about the mechanisms that aid in dealing with those anthropic and natural stressors. Hence, it is of utmost importance to understand how environmental variability affects mussel physiology, responses, and acclimatization in changing environments in the future, and to facilitate determining risk for other organisms in the same ecosystems.

Gene expression profiling has emerged as a powerful tool to investigate physiology, providing an expanded view of the molecular changes that occur when organisms experience environmental stress and understand responses before establishing phenotypic changes. In this contest, we have employed a set of core genes actively involved in different mussel physiological functions related to stress response and acclimatization.

The general aim of this research project is to investigate the molecular mechanisms governing some acclimatory and stress responses of the Mediterranean mussel (*Mytilus galloprovincialis)* by addressing relevant life stages and environmental stressors of emerging

concern. Specifically, the experimental approach was constructed into 2 phases based on their life stages namely larval and adult stages to accomplish the following specific objectives

Phase 1- Larval stages

Specific objective: Assessing relevant physiological processes across mussel embryo development and the possible adverse effects triggered by plastic pollution

Phase 2- Adult stages

Specific objective: Studying basal expression of transcript involved in physiological processes of adult mussels in relation with gender and reproduction bias under natural gradients of physical/chemical parameters

Larval stages are considered more vulnerable to environmental threats than adult stages and therefore are addressed as the critical phase in a bivalve lifecycle. In their natural environment, larvae may be exposed to an array of natural or human-made stress factors potentially affecting the regular developmental program, thus impairing their fitness when adults (for example, by impairing growth rates, shell biogenesis, reproductive output), or even reducing their survival, with negative effects on population dimension (Balbi et al., 2016, 2017; Estévez-Calvar et al., 2017; Wu et al., 2016; Xu et al., 2016). Understanding how larvae respond to such environmental pressures is essential to link the population shifts projected by habitat quality models with the underlying molecular mechanisms that drive physiological impairments.

When considering adult mussels, much is known about their physiological adjustments to changes in environmental parameters including temperature, pH, and salinity, as well as pollutants under laboratory conditions (Dowd and Somero, 2013; Martinez et al., 2019; Navarro et al., 2012). In contrast, less evidence is available regarding physiological variability in their natural environments and whether this may be driven by either variability

of environmental parameters or endogenous factors, such as gender bias and reproduction. This is a relevant knowledge gap to predict animal fitness facing projected environmental trends.

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Chapter 1

Assessing relevant physiological processes across mussel embryo development and the possible adverse effects triggered by plastic pollution

During the first and second year, I worked on embryo/larval stages of *Mytilus galloprovincialis* by analyzing transcription of genes involved in relevant protective functions across early larvae development and under different physiological conditions.

As a preliminary analysis, basal transcription of genes involved in the lysosomal system, immune response, and those encoding metallothioneins (MT) under physiological growth was inferred collecting embryos at 24 h post-fertilization (pf), when the first free-swimming trochophore larvae are developed, and at 48 h pf, when the first fully developed D-shape shelled veliger is formed. Results (Fig 1) corroborated previous hypotheses of a general reinforcement of protective systems needed to counteract the increased exposure to environmental stressors.

In mussels, the lysosomal system is involved in the food digestion, cellular defense mechanisms, sequestration and detoxification of xenobiotics (Bocchetti and Regoli, 2006). Mussel larvae are supposed to acquire a suitable lysosomal system at the D-veliger stage when they get a fully developed digestive system, hence increase their dirct interaction with the surrounding environment (Franzellitti et al., 2019a). Phagocytosis, antimicrobial peptide production and chemical defenses have been proposed as immune mechanisms in veliger stage (Dyrynda et al., 1995), and emerging of immune mechanisms at the early larval stages supposed to aid larvae to protect from pathogens (Balseiro et al., 2013; Bassim et al., 2015). MTs are involved in controlling cellular homeostasis of metals, protecting against metals and oxidant damage, metabolic regulation, sequestration and/or redox control (Mao et al., 2012), and supposed to be involved in the early larval stages to deal with

stressors arise due to metals, and the survivability of progeny (Mao et al., 2012; Weng and Wang, 2014).

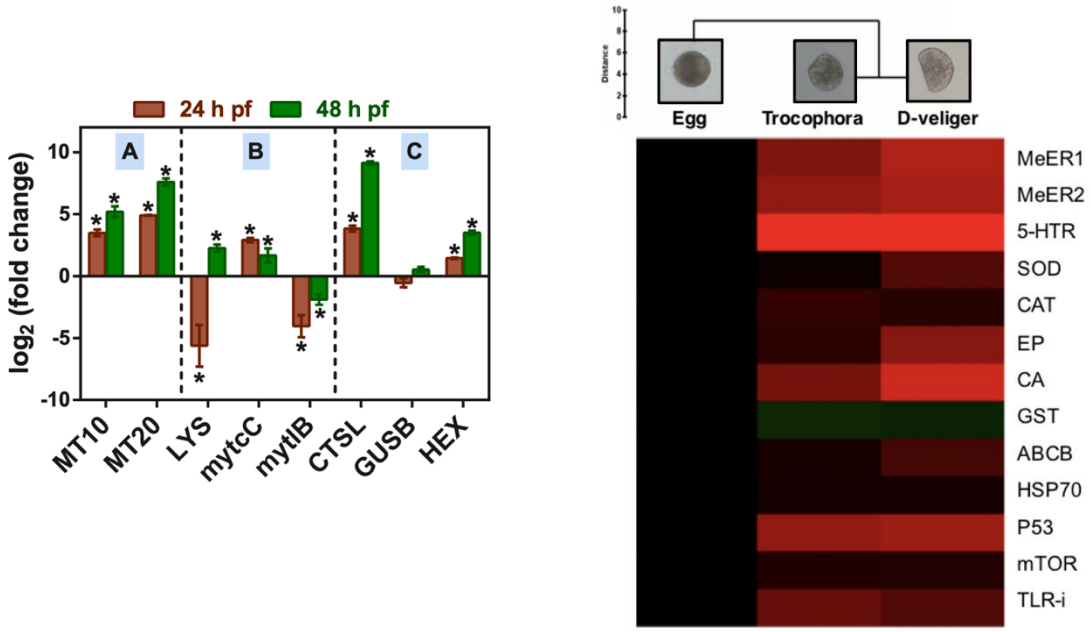

Fig 1. Transcriptional profiles of genes involved in (A) antioxidant response (metallothionein 10, MT10, metallothionein 20, MT20), (B) lysosomal response (cathepsin L, CTSL; ß-glucuronidase, GUSB; hexosaminidase, HEX), (C) immune response (lysozyme, LYS; mytilin b, MYTLb; myticin c, MYTCc) during early larval development in mussels agree with the general fast development of protective systems as showed by the heatmap (D). Gene transcription was evaluated by qPCR in mussel embryos grown under physiological conditions up to 24 h and 48 h pf. Data are log_2-transformed relative expressions with respect to unfertilized eggs (mean ± SEM; N = 12). *$p < 0.05$ vs. unfertilized eggs; Mann-Whitney U-test (adapted from Franzellitti et al. (2019) and Balbi et al.(2016)).

I further implemented experiments to address transcriptional regulation in response to plastic pollution, here represented by the styrene monomer (henceforth addressed to as styrene), as a possible component plastic leachate (Andrady, 2011; Thaysen et al., 2018), and polystyrene microplastics. These research activities have been performed within the Joint Program Initiative (JPI) Oceans project PLASTOX (Direct and indirect ecotoxicological impacts of microplastics on marine organisms; http://www.jpi-oceans. eu/plastox).

Other than major publications on this project, two review papers entitled “Microplastic exposure and effects in aquatic organisms: A physiological perspective” and the magazine article “Silent Killer in the ocean” were published. Both articles explain the microplastic in the marine water and the consequences on marine organisms and are reported as **Supplements 1, 2.**

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Styrene impairs normal embryo development in the Mediterranean mussel (*Mytilus galloprovincialis*)

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ABSTRACT

This study analysed the effects of styrene, a main monomer in plastic manufacturing and acknowledged to be amongst the most common plastic leachates, on early embryo development of the Mediterranean mussel. Embryotoxicity tests showed that styrene impaired normal embryo development at concentrations (0.01 µg/ L–1 mg/L) encompassing the environmental range. Occurrence of normal D-veligers was significantly reduced up to 40% of the total, and larval size was reduced of about 20%. D-veligers grown in the presence of styrene (0.1 and 10 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 (ABCB) and Mrp (ABCC), the two main ABC transporters of embryonal MXR system. Indeed, ABCB transcription was not affected by styrene, while ABCC was up-regulated. At these same concentrations, transcriptional profiles of 15 genes underlying key biological functions in embryo development and potential targets of adverse effects of styrene were analysed. Main transcriptional effects were observed for genes involved in shell biogenesis and lysosomal responses (downregulation), and in neuroendocrine signaling and immune responses (up-regulation). On the whole, results indicate that styrene may affect mussel early development through dysregulation of gene transcription and suggest the possible conservation of styrene mode of action across bivalve life cycle and between bivalves and humans, as well as through unpredicted impacts on protective systems and on shell biogenesis.

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1. Introduction

There is no doubt that nowadays human life could not be imagined without plastics, since plastic materials offer many benefits to humanity by providing tools that cover every sector of human life, from health and food preservation to transportation and enhancing digital age (UNEP, 2016). The main fraction of litter discarded into the aquatic environments consist of plastic materials, and between 4.8 and 12.7 million tons of plastic had found its way into the oceans (UNEP, 2016).

Discarded plastic debris is washing up onto the shores of countries throughout the world (Convey et al., 2002; Fauziah et al., 2015; Hidalgo-Ruz and Thiel, 2013; Kordella et al., 2013; Semeoshenkova and Williams, 2011; Watts et al., 2017), and is spread over long distances by winds, ocean currents, waves and tidal action (Cózar et al., 2014; Obbard et al., 2014). In this process, under the effects of thermal UV degradation and hydrolysis, chemical weathering and mechanical processes, plastic debris gradually becomes brittle (GESAMP, 2015), and leaching of dissolved chemicals is observed, leading to consider plastic debris as a new source of global contamination for the marine environment (Kwon et al., 2018, 2017, 2015, 2014).

The majority of research on marine plastic debris has focused on the effects of macro-plastics, including entanglement and ingestion hazards posed to wildlife (Galloway and Lewis, 2016). Other studies also demonstrated that abundance and biological impacts of micro-plastics and micro-fibers may be just as damaging (Andrady, 2011; Moore, 2008), and increasing concern is addressed to nano-scale plastic debris, due to its longer retention time in the digestive systems of organisms, and its unique properties and biological reactivity that are likely to enhance its toxicity (Canesi and Corsi, 2016; Cole and Galloway, 2015). Studies addressing the biological impacts of leachates from plastics on marine organisms are under-represented in the literature. Common examples of chemicals released through plastic leaching include bisphenol A (BPA), styrene oligomers, phthalates, and vinyl chlorides. Some of these may act as endocrine disruptors, others are carcinogens, and some have not yet been classified (Barnes et al., 2009; Hirai et al., 2011). Recent investigations highlighted the sensitivity of early embryonal stages of the Mediterranean mussel (*Mytilus galloprovincialis*) towards the adverse effects of BPA, which impaired normal larval development within a range of environmentally realistic concentrations (Fabbri et al., 2014), disrupted the processes of shell biogenesis, and deregulated gene transcription (Balbi et al., 2016).

This study analyzes the potential embryotoxicity of styrene monomer (henceforth addressed to as styrene) in Mediterranean mussels. Styrene is one of the most important chemical commodity for manufacturing polymers employed in a wide range of applications, including plastic manufacturing, latex paint and coating production, synthetic rubbers, polyesters and styrene-alkyd coatings. In the marine environment, styrene oligomers were detected worldwide at concentrations up to 30 μg/L in surface coastal waters (Kwon et al., 2015), and recently levels ranging from 0.31 to 4.31 μg/L have been reported in deep seawaters of the North-West Pacific Ocean (Kwon et al., 2017). Styrene is listed amongst the Hazardous and Noxious Substance (HNS) cargoes in the main EU Atlantic ports (Tornero and Hanke, 2016). Indeed, styrene is genotoxic and carcinogenic to humans (Huff and Infante, 2011), even though it is only moderately toxic to fish, daphnids, and amphipods (Cushman et al., 1997). Furthermore, some styrene oligomers are suspected to be estrogenic and endocrine disruptor compounds, although the real extent of these effects is under scientific debate (Gelbke et al., 2015).

In vivo experiments with blue mussels (*Mytilus edulis*) showed that styrene may impair the lysosomal system and cause increased DNA damage (Mamaca et al., 2005), as well as altered gene transcription (Diaz de Cerio et al., 2013) in exposed animals. Although these studies disclosed relevant effects of styrene on mussel physiology, no information is available on the early developmental stages, which are vulnerable to a wide array of environmental threats, including plastic polymers (Balbi et al., 2017; Capolupo et al., submitted; Cole and Galloway, 2015).

In this study, fertilized eggs were exposed to styrene in a wide concentration range (from 0.01 µg/L to 1 mg/L), and potential impairment of percentage of normally developed larvae, larval size and morphology was assessed. Cytoprotective responses by means of analysis of Multixenobiotic resistance (MXR) efflux activity and related gene transcriptions were investigated, as MXR-related transporters may be involved in biotransformation pathways leading to styrene metabolism (Andersen et al., 2017), or their expression affected by interaction of styrene with mediators of cell signaling pathways that may be involved in their transcriptional control (Andersen et al., 2018, 2017). To gain deeper insight into potential styrene effects on embryos, transcriptional changes of further 15 target genes were also assessed. According to our recent studies on emerging contaminants (Balbi et al., 2017, 2016), a "core" set of genes was selected. This core gene set included genes involved in shell biogenesis and neuroendocrine modulation, and underpins known biological functions regulated in the development of early embryo under physiological conditions. Their differential regulation may disclose putative interaction of styrene with normal developmental processes of mussels. Furthermore, we also included the analysis to transcripts of genes involved in lysosomal, immune and antioxidant responses, which have been previously addressed to as potential targets of styrene effects in mammals and in adult mussels (Andersen et al., 2017; Diaz de Cerio et al., 2013; Mamaca et al., 2005). The whole transcriptional approach attempted to disclose whether known responses to styrene exposure derived from studies on mammals and adult mussels can be detected also in mussel embryos, and whether (unpredictable) responses related to the specific physiological traits of embryos, (likely) related to the embryotoxic potential of the compound can be observed.

All investigations were performed at 48 h post fertilization (pf), when the first fully developed shelled veliger (D-veliger) is formed and critical improvements of larval physiology take place (Bassim et al., 2014).

2. Methods

2.1. *Animal holding, rearing and treatment of larvae*

Sexually mature Mediterranean mussels (*Mytilus galloprovincialis*) were obtained from a government certified mussel farm (Cooperativa Copr.al.mo, Cesenatico, Italy), transferred to the laboratory, and acclimatized in static tanks containing aerated 35-psu artificial seawater (ASW) at 16 °C (ASTM, 2004). Gamete collection, oocyte fertilization and handling of larvae were performed as reported by Fabbri et al. (2014). Briefly, when mussels begun to spontaneously spawn, each individual was immediately placed in a 250-mL beaker containing 200 mL of aerated ASW until complete gamete emission. Sperm and eggs were sieved through 50 µm and 100 µm meshes, respectively, to remove impurities. Egg quality (shape, size) and sperm motility were checked using an inverted light microscope. Eggs were fertilized with an egg:sperm ratio 1:10 in 96-well (embryotoxicity test), 12-well (MXR activity), or 6-well (RNA extraction) cell culture plates. After 30 min, fertilization success (n. fertilized

eggs/n. total eggs × 100) was verified microscopically (> 85%).

Styrene was purchased as a liquid with a nominal concentration of the stock solution 0.906 g/mL (> 99% purity; CAS number 100-42-5; Sigma Aldrich, Milan, Italy). Working solutions in ASW were freshly prepared at 10X concentrations tested in the embryo treatments and maintained under continuous stirring in lid-closed glass bottles. Concentrations of the working solutions were verified by GC–MS. At 30 min post fertilization (pf), styrene was added to fertilized eggs in the proper wells from the working solutions suitably diluted to reach the final nominal concentrations tested.

For the embryotoxicity assay, a wide range of nominal concentrations from 0.01 µg/L to 1 mg/L was selected. An EC50 (defined as the toxicant concentrations causing 50% reduction on percentage of normal D-larvae) value of 1.1 ± 0.4 µg/L was estimated using a four-parameter logistic curve fitting approach with the GraphPad Prism 6 software package (GraphPad Inc.). Goodness-of-fit tests were performed by the GraphPad software as a default operation to test for statistical adequacy of the employed fitting model. Treatments for MXR activity and mRNA expression analyses were carried out at 0.1 µg/L (about 0.1X EC50 and equal to LOEC, lowest observed effective concentration in the embryotoxicity test; Fig. 1A) and 10 µg/L (about 10X EC50 and equal to 100X LOEC) nominal styrene concentrations. As styrene is volatile, each plate was closed by its lid. A control group (i.e. untreated embryos) was maintained in parallel to the experiment. Embryos were grown at 16 °C ± 1 °C for 48 h in the presence of styrene at the selected concentrations. Data for all endpoints are derived from 4 independent experimental replicates (N = 4). Further technical replicates within each experiment have been performed (according to the endpoint analysed), and details are reported in the figure captions.

2.2. *Embryotoxicity assay and morphometric analyses*

The 48-h embryotoxicity test (ASTM, 2004) was carried out according to Fabbri et al. (2014). At the end of the incubation time, samples were fixed with buffered formalin (4%). All larvae in each well were examined and photographed using an inverted light microscope (Axiovert 100, Zeiss, Milan, Italy) equipped with a CCD UC30 camera (Nikon) and a digital image acquisition software (NIS Elements imaging software, Nikon). Observations were carried out by an operator unknowledgeable of the experimental conditions. A larva was considered normal when the shell was D-shaped (straight hinge) and the mantle did not protrude out of the shell, and malformed if had not reached the stage typical for 48 h pf (trocophore or earlier stages) or when some developmental defects were observed (concave, malformed or damaged shell, protruding mantle). The recorded endpoint was the percentage of normal D-veligers with respect to the total in each well, including malformed larvae and pre D-veliger stages. The acceptability of test results was based on controls for a percentage of normal D-shell stage larvae > 75% (ASTM, 2004). Area occupied by larvae (calculated as pixels) was assessed using the ImageJ software according to Cole and Galloway (2015).

2.3. MXR activity assay

The assay was based on the analysis of cell-based transport using rhodamine 123 (Rho123, Sigma Aldrich, Milan, Italy), a fluorescent model substrate for the main MXR-related transporters (Smital et al., 2000). Rho123 shows low to moderate rates of passive membrane permeation, so that it is effectively extruded by ABC efflux pumps (Luckenbach et al., 2014). Therefore, the amount of Rho123 in the cell is a measure of transporter activity: high activity of transporters is indicated by a weaker Rho123

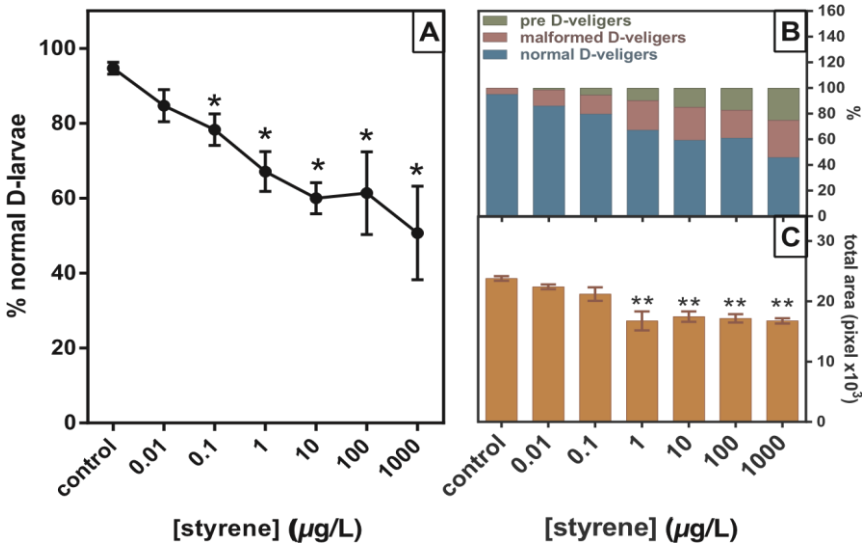

Fig. 1. Effects of different concentrations of styrene (0.01–1,000 µg/L) on M. galloprovincialis normal larval development. (A) Results of the 48-h embryotoxicity assay. Data are reported as mean ± SEM of 4 experiments carried out in 96-multiwell plates (6 replicate wells for each sample) (N = 4). (B) Percentage of normal D-veligers, malformed D-veligers and pre-veligers (non-fully formed veligers, trocophorae, and non-developed oocytes) in each experimental treatment. For each group, pairwise statistical comparisons amongst styrene treatment levels and control are reported in Table 1. (C) The average size (mean ± SEM; N =4), measured as pixels (ImageJ) of larvae following the 48-h exposure to styrene. *p < 0.05 vs control; **p < 0.01 vs control.

fluorescence signal, whereas a stronger fluorescence signal corresponds to lower transporter activity. A co-exposure in vitro assay was performed to assess the effects of styrene on MXR transport activity as reported in detail by Franzellitti et al. (2017). Rho123 (prepared in dimethylsulfoxide, DMSO) was added to 48 h pf control and treated embryos to reach the assay concentration of 2.5 µM. DMSO concentrations never exceed 0.1% v/v. Embryos were incubated for 90 min at 16 °C in the dark in the presence of styrene and Rho123. The duration of exposure and Rho123 concentration were selected during preliminary experimental trials, which showed that these conditions did not significantly affect embryo viability while providing the most stable and repeatable fluorescence readings (data not shown). At the end of the incubation period, samples have been processed according to Franzellitti et al. (2017). Fluorescence measurements were performed using a Jasco FP-6200 fluorometer (λexcitation = 485 nm; λemission = 530 nm). Values were normalized to total protein content using the Qubit protein assay with the Qubit 2.0 system (Thermo Fisher, Milan, Italy) according to the manufacturer's instructions. Results were expressed as mean ± SEM of the percentage of variation relative to controls

2.4. *RNA extraction and qPCR analyses*

Total RNA extraction from mussel embryos was performed following procedures described by Balbi et al. (2016). RNA concentration and quality were verified using the Qubit RNA assay through the Qubit 2.0 system (Thermo Fisher, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 1 µg total RNA using the iScript supermix (Bi-orad Laboratories, Milan, Italy) following manufacturer's instructions.

Primer pairs and protocols employed for quantitative real time PCR (qPCR) assays are reported in previous studies (Balbi et al., 2017, 2016; Capolupo et al., Capolupo et al., submitted). A preliminary stability analysis of 6 established candidate reference transcripts was performed through different computational methods in order to achieve a robust normalization of qPCR data (Balbi et al., 2016; Franzellitti et al., 2015). The pair helicase/elongation factor 1α was the best performing combination of reference genes to be employed for qPCR data normalization. Calculations of relative expression of target mRNAs was performed by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne software tool (Thermo Fisher, Milan, Italy). Data were reported as mean ±

SEM of relative expression (log_2-transformed fold changes) with respect to controls.

2.5. *Statistics*

Data from embryotoxicity assays, morphometric analyses, and rhodamine efflux assay were analysed using non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney U-test, after deviations from parametric ANOVA assumptions were observed in appropriate tests (Normality: Shapiro-Wilk's test; equal variance: Ftest). These statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Inc.). qPCR data were evaluated with the REST software (Pfaffl et al., 2002) that uses a randomisation test with a pairwise reallocation to assess the statistical significance of the differences in expression between each treatment-exposed group and the controls. Further comparisons between pair of treatments were performed using the Mann-Whitney U-test. In all approaches, $p < 0.05$ was set as the threshold level of statistical significance.

Data from qPCR investigations were further submitted to permutation multivariate analysis using the PRIMER v6 software (Anderson et al., 2008). Log_2-transformed fold change variations of the target transcripts were used to calculate similarity matrices based on the Euclidean distance (999 permutations), and a distance-based redundancy linear modeling (DISTLM) with a test of marginality was also performed to account for the contributions of the functional groups of transcripts in explaining the total observed variance.

3. Results

3.1. *Effects of styrene on embryo development*

Fertilized eggs were exposed to different concentrations (from 0.01 µg/L to 1 mg/L) of styrene in 96-microwell plates, and the percentage of normal D-veliger larvae was evaluated after 48 h pf (Fig. 1A). Styrene induced a significant decrease in normal larval development from 0.1 µg/L ($p < 0.05$), with percentages decreasing thereafter (Fig. 1A). Using a four-parameter logistic curve fitting approach, an EC50 value of 1.1 ± 0.4 µg/L (mean ± SD) was estimated. As shown in Fig. 1B, styrene mainly induced the development of malformed D-veligers ($p < 0.05$, Table 1), although a delay in development was also observed, with an increased presence of embryos at the pre-veliger stage at the higher concentrations tested (Fig. 1B; $p < 0.05$, Table 1).

No significant mortality due to styrene was observed, since at 48 h pf the average number of viable larvae per well did not differ significantly between controls and styrene treatments (data not shown). A significant reduction in the size of styrene-exposed D-veligers was observed from 1 µg/L styrene (Fig. 1C), with samples stably showing an average 20% reduction of larvae area compared to controls.

3.2. *Effects of styrene on MXR efflux activity and ABCB/ABCC mRNA expression*

Changes of MXR transport activity in mussel D-veligers was investigated by assessing cell accumulation of the model

Table 1
Pairwise comparisons of percentages of malformed D-veligers or pre D-veligers between different styrene treatment levels vs controls according to the twotailed Mann-Whitney U test (N =4). Statistical differences are highlighted in bold.

	Malformed D-veliger		**Pre D-veliger**	
	t	**p**	**t**	**p**
Control vs 0.01 µg/L	2.26	0.06	1.40	0.453
Control vs 0.1 µg/L	**3.19**	**0.026**	**6.37**	**0.036**
Control vs 1 µg/L	**3.01**	**0.035**	**3.14**	**0.039**
Control vs 10 µg/L	**5.10**	**0.036**	2.97	0.144
Control vs 100 µg/L	**3.65**	**0.009**	**3.28**	**0.039**
Control vs 1000 µg/L	**4.52**	**0.008**	**7.41**	**0.009**

fluorescent substrate Rho123 at 0.1 and 10 µg/L styrene concentrations, representing the 0.1X and 10X of the mean EC50 assessed through the embryotoxicity assays (Fig. 2A). Compared to controls, Rho123 accumulation was increased by both styrene treatments that indicates a significantly reduced MXR activity (Fig. 2A). Under the same treatments, levels of the ABCB transcript (encoding Pgp) were unchanged, (Fig. 2B) while ABCC (encoding Mrp) transcripts were up-regulated (Fig. 2C).

3.3. *Effects of styrene on gene transcription*

The effects of embryo exposure to styrene (0.1 or 10 µg/L) on transcription of 15 genes involved in different physiological functions was evaluated in D-veligers (48 h pf). Full gene names are reported in caption to Fig. 3. All transcripts involved in the antioxidant response (CAT, GST, SOD) showed no significant variations following styrene treatments (Fig. 3A). MYTLb and MYTCc, encoding two antimicrobial peptides involved in the mussel immune response, were significantly up-regulated at 0.1 µg/L styrene (MYTLb) and/or at 10 µg/L styrene (MYTLb, MYTCc) (Fig. 3B). The LYS transcript was significantly downregulated at 10 µg/L styrene (Fig. 3B). A significant down-regulation was observed for all transcripts encoding lysosomal enzymes at both styrene concentrations (Fig. 3C). Transcripts involved neuroendocrine signaling were significantly up-regulated (MeER1 and MeER2) or downregulated (5-HT1) in D-veligers grown in the presence of 10 µg/L styrene (Fig.3D). As shown in Fig. 3E, styrene induced significant modulation of transcripts involved in shell biogenesis, with down-regulation of CS and CA at both styrene concentrations tested, and upregulation and down-regulation of EP at 0.1 and 10 µg/L styrene, respectively.

DISTLM analysis by functional group revealed that expression patterns of transcripts involved in shell biogenesis and lysosomal responses mostly explained the observed effects of styrene in D-veligers, with further significant contributions being provided by the MXR system and neuroendocrine signaling (Fig. 4).

4. Discussion

Embryotoxicity tests performed in this study showed that styrene may significantly alter normal embryo development of Mediterranean mussels in a wide concentration range, the main observed effects being reduction of larval size at 48 h post fertilization (pf) and increased occurrence of malformations. Although a percentage value of normal D-larvae below 50% (as those observed at highest concentrations tested) must be regarded as an indication of severe physiological impairment of embryo, lack of significant increase of mortality in styrene-treated embryos indicates that the concentrations of the compound use were sub-lethal towards mussel embryos.

Data from embryotoxicity tests agree with previous reports on developmental effects of

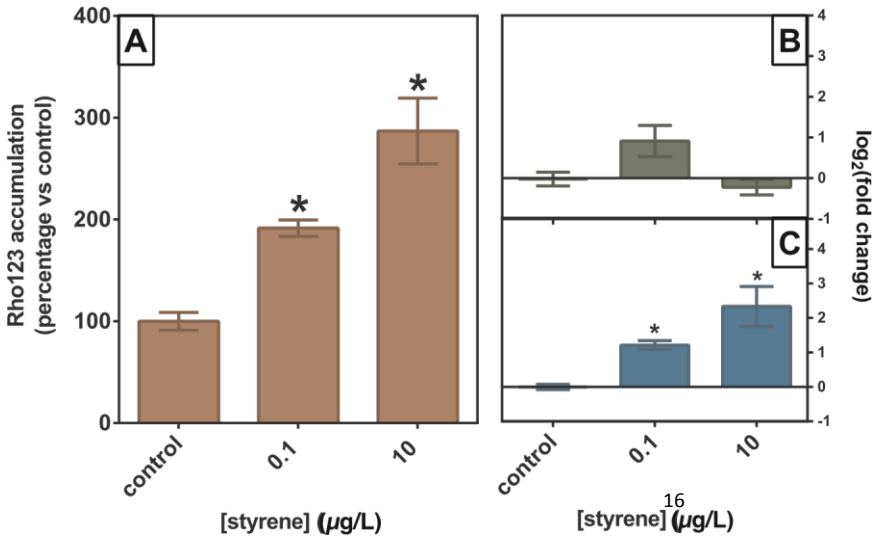

Fig. 2. Effects of styrene on the MXR system in embryos of *M. galloprovinicialis* at 48 h pf (D-veliger stage). Embryos were grown for 48 h in the presence of 0.1 µg/L or 10 µg/L styrene. (A) Rho123 accumulation. Data are expressed as mean ± SEM of 4 experiments carried out in 12- or 6multiwell plates (3 replicate wells for each sample) (N = 4) of the variation vs respective controls (ctr; untreated embryos). (B) ABCB (encoding the P-glycoprotein, Pgp) and (C) ABCC (encoding the Multidrug resistance related protein, Mrp) expression changes. Data are expressed as mean ± SEM (N =4) of the relative variation (log_2-normalized fold changes) vs controls (untreated embryos) (*p < 0.05).

polystyrene nanoparticles in the Mediterranean mussel (Balbi et al., 2017) and in the sea urchin *Paracentrotus lividus* (Della Torre et al., 2014), suggesting that chemical composition per *se* other than structure (monomeric vs polymeric forms) may explain embryotoxicity potential of styrene-based compounds.

It is worth noting that within the same concentration range and a similar exposure setup (i.e. number of exposed embryos, treatment volume and duration) tested in the present study, 50 nm NH2-polystyrene nanoparticles tested by Balbi et al. (2017) mainly induced larval malformations, while delays in development were observed at higher concentrations. Therefore, at these concentrations interaction of styrene-based compounds with processes of shell biogenesis can be hypothesized.

D-veligers are suitable models to infer stress responses in mussel embryos, since they represent a critical stage in the ontogeny of some protective systems (Franzellitti et al., 2017; Hamdoun and Epel, 2007; Moreira et al., 2018), and are sensitive to the adverse effects of emerging pollutants (Balbi et al., 2017, 2016; Estévez-Calvar et al., 2017). The Multixenobiotic Resistance (MXR) system is considered a general and broad spectrum protective mechanism that consists of membrane and intracellular active transporters providing an active first-tier defense against environmental chemicals to prevent their accumulation and toxic effects (Bielen et al., 2016). A recent study that characterized expression and activity of MXR-related transporters across early development of mussel supported the hypothesis that in embryos as in adults this system plays a general broad-spectrum protective role against toxicants (Franzellitti et al., 2017). Data reported in this study showed the increased accumulation of the model fluorescent substrate Rho123 in styrene-treated D-veligers, which suggests a reduction of MXR total efflux activity. This effect appears not linked to the transcriptional regulation of the genes encoding the two main MXR-related ABC transporters Pgp (ABCB) and Mrp (ABCC), both effectively supporting total MXR activity in D-veligers (Franzellitti et al., 2017). Indeed, ABCB was unaffected, while ABCC was significantly up-regulated in styrene-treated embryos compared to controls. This finding may lead to hypothesis that styrene acts as a MXR substrate causing Rho123 accumulation, without related transcriptional modulation. It is known that changes in efflux activity do not always reflect transcriptional profiles of genes encoding ABC transporters. This is probably due to the complex regulation of the production and activity of ABC transporters involving transcriptional, post-transcriptional (splicing, regulation of mRNA stability), and post-translational (phosphorylation, glycosylation, ubiquitination, protease-mediated degradation) processes, as well as from the complexity of substrate – transporter interactions (Hennessy and Spiers, 2007; Scotto, 2003). We recently showed that the pharmaceuticals propranolol, carbamazepine, and fluoxetine, which are potential substrates for MXR transporters, affected mussel ABCB and/or ABCC transcription along with changes in MXR efflux activity (Franzellitti et al., 2016), indicating that exposure to contaminants acting as substrates/inhibitors of ABC pumps may lead to an altered transcription. The observed positive regulation of ABCC transcription might represent a feedback response to increased requirements for novel Mrp transporters mediating styrene extrusion. Indeed, it is known that in mammalian cells styrene undergoes metabolism by oxidation through the action of several cytochromes (Andersen et al., 2017), and that at least 1% of styrene biotransformation is elicited by its conjugation with GSH through the glutathione-s-transferase (GST) enzyme activity (Carlson, 2011; Rueff et al., 2009). Therefore, ABCC up-regulation may result from the activation of specific biotransformation pathways in which Mrp transporters constitute the phase III (i.e.

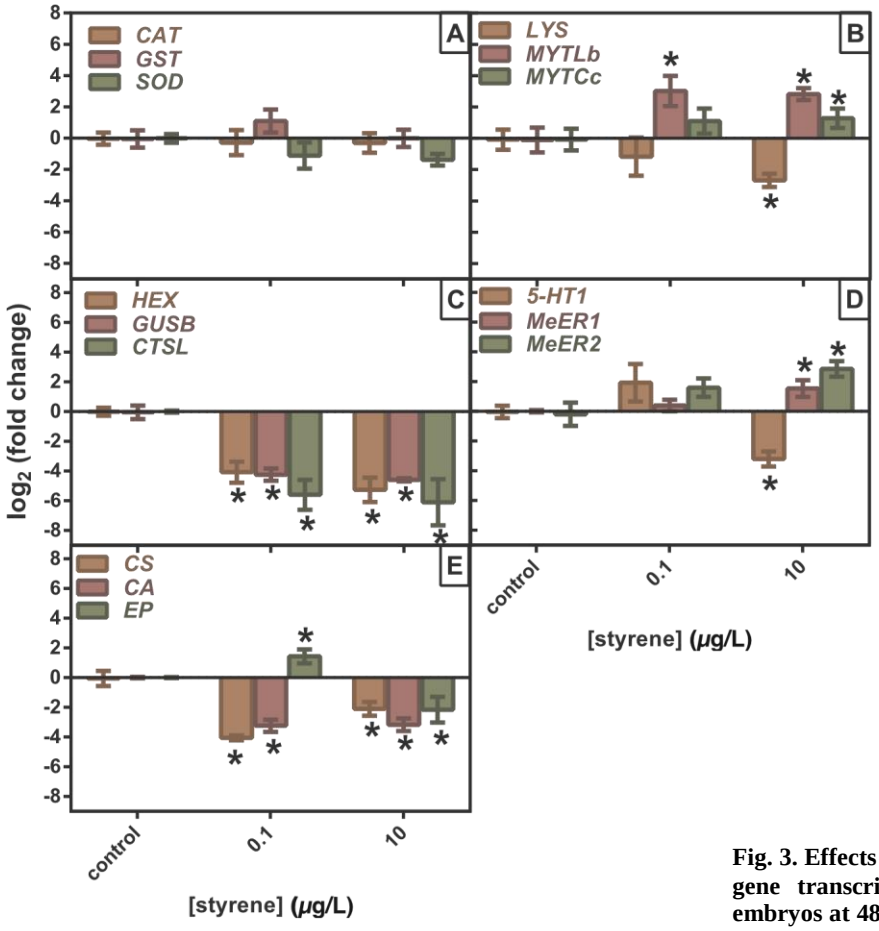

Fig. 3. Effects of styrene (0.1 and 10 µg/L) on gene transcription in *M. galloprovincialis* embryos at 48 h pf (D-veliger stage). Relative expression of transcripts involved in (A) antioxidant responses (genes encoding catalase, CAT; glutathione s-transferase, GST; superoxide dismutase, SOD), (B) immune responses (genes encoding lysozyme, LYS; mytilin b, MYTLb; myticin c, MYTCc), (C) lysosomal system (genes encoding cathepsin L, CTSL; ß-glucuronidase, GUSB; hexosaminidase, HEX), (D) neuroendocrine signaling (genes encoding type 1 serotonin receptor, 5-HT1; type 1 estrogen receptor, MeER1; type 2 estrogen receptor, MeER2), (E) shell biogenesis (genes encoding carbonic anhydrase, CA; chitin synthase, CS; extrapallial protein, EP). Data are reported as mean ± SEM of 4 experiments carried out in 6-multiwell plates (3 replicate wells for each sample) (N = 4). Values represent the relative variation (log_2-normalized fold changes) vs controls (untreated embryos) (*$p < 0.05$).

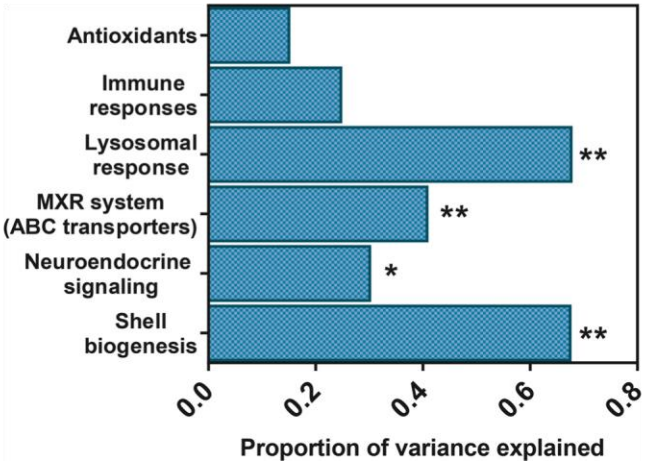

Fig. 3. Effects of styrene (0.1 and 10 µg/L) on gene transcription in *M. galloprovincialis* embryos at 48 h pf (D-veliger stage). Relative expression of transcripts involved in (A) antioxidant responses (genes encoding catalase, CAT; glutathione s-transferase, GST; superoxide dismutase, SOD), (B) immune responses (genes encoding lysozyme, LYS; mytilin b, MYTLb; myticin c, MYTCc), (C) lysosomal system (genes encoding cathepsin L, CTSL; ß-glucuronidase, GUSB; hexosaminidase, HEX), (D) neuroendocrine signaling (genes encoding type 1 serotonin receptor, 5-HT1; type 1 estrogen receptor, MeER1; type 2 estrogen receptor, MeER2), (E) shell biogenesis (genes encoding carbonic anhydrase, CA; chitin synthase, CS; extrapallial protein, EP). Data are reported as mean ± SEM of 4 experiments carried out in 6-multiwell plates (3 replicate wells for each sample) (N = 4). Values represent the relative variation (log_2-normalized fold changes) vs controls (untreated embryos) (*$p < 0.05$).

extrusion of GSH-conjugated products of phase II; Leslie et al., 2005). Nevertheless, according to Hamdoun and Epel (2007) toxicant transformation by the cytochromes should not appear until tissue differentiation.

To the best of our knowledge, no data on styrene interacting with ABC transporters are currently available, therefore no comparative evaluations with previous reports can be performed. Balbi et al. (2017) showed significant ABCB down-regulation in mussel D-veligers grown in the presence of 150 μg/L 50 nm NH2-polystyrene nanoparticles. However, the MXR efflux activity was not measured, thus a relationship of the transcriptional effect with a functional impairment was not established. On the whole, these data highlight the capacity of styrene to impair the MXR system in D-veligers. Besides conferring chemioresistance to embryos and adults, MXR-related transporters, in particular Mrp, are thought to be integral to the developmental processes (Franzellitti et al., 2017); therefore, this effect may be physiologically demanding for embryos.

To gain further insights into the possible mechanisms behind styrene effects in developing embryos, expression changes of further 15 transcripts were analyzed. These transcripts are involved in shell biogenesis, lysosomal responses, neuroendocrine signaling, immune responses, and antioxidant responses. Main effects were observed for genes involved in shell biogenesis and lysosomal responses (downregulation), and in neuroendocrine signaling and immune responses (up-regulation).

The biomineralization process in marine organisms involves mechanisms of organic matrix formation by synthesis/secretion of macromolecules as well as of ion transport (Ca^{2+}, HCO_3^- and H^+) to the mineralizing sites (Allemand et al., 2004; Johnstone et al., 2015). In adult mussels both mechanisms are mediated by a network of enzymes and macromolecules synthetized and secreted from the dorsal mantle, which regulates calcification rates and shell morphology (Kocot et al., 2016; Marie et al., 2012). Carbonic anhydrases (CAs) play a major role in ion transportation to provide the source of HCO3 – for CaCO3 precipitation (McDougall and Degnan, 2018; Wang et al., 2017). Secreted macromolecules consist of insoluble components, that provide the framework and mechanical properties to the shell, and soluble proteins involved in crystal nucleation and growth (Belcher et al., 1996; Falini et al., 1996). Chitin, an insoluble polysaccharide, forms the structural framework of mollusk shells, and is one of the key players in driving and coordinating the mineralization processes and in determining shell morphology (Falini and Fermani, 2004). Chitin synthase (CS) belongs to the pool of enzymes responsible for the synthesis and translocation of chitin (Weiss et al., 2006). The extrapallial protein (EP) is a soluble acidic calcium binding protein that regulates the production of different polymorphs of calcium carbonate (Hattan et al., 2001; Yin et al., 2009).The concomitant down-regulation of transcripts encoding these proteins, in particular CS, observed in this study may underpin the increased percentage of malformed D-veligers in styrene-treated embryos and the decreased embryo size showed by morphometric analyses. This finding suggests the dysregulation of shell-biogenesis induced by styrene. It is worth noting that similar transcriptional outcomes were observed in mussel D-veligers grown in the presence of 150 μg/L 50-nm NH2-polystyrene nanoparticles, which also showed increased occurrence of shell malformations and reduced calcification (Balbi et al., 2017).

Lysosomes have been showed as potential targets for styrene toxicity in mussels. Indeed, reduction of lysosomal membrane stability both in haemocytes (Mamaca et al., 2005) and in digestive cells (Ruiz et al., 2014) was previously reported in mussels in vivo exposed to styrene under laboratory conditions. In agreement, in this study a dramatic down-regulation of transcripts encoding lysosomal enzymes was observed in styrene-treated D-veligers, suggesting the onset of a

dysfunctional lysosomal condition. In particular, β-glucuronidase (GUSB) and hexosaminidase (HEX) are the lysosome enzymes employed as markers to determine changes in lysosomal size and membrane stability, respectively (Izagirre et al., 2009; Marigómez et al., 2005), and down-regulation of GUSB and HEX transcripts was observed in mussel digestive cells suggesting a decrease in lysosomal membrane stability and loss of lysosomal functionality (Izagirre et al., 2014). Pending data on the function and the dynamic of the lysosomal system across mussel development, we may highlight that impairment of lysosomal functions has been related to reduced scope for growth in bivalves and animal health status that could predict consequences at the population level (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 styrene-based compounds, being observed in mussel adults in vivo exposed to 3–5 ppm styrene for 3 days (Diaz de Cerio et al., 2013), and in embryos exposed to different concentrations of nano-size and micro-size polystyrene particles (Balbi et al., 2017; Capolupo et al., submitted). It can be hypothesized that styrene may challenge immune defenses of mussels as it does in mammalian models, where immune and inflammatory responses are well documented (Niaz et al., 2017; Röder-Stolinski et al., 2008).

Conflicting evidence are reported on the potential interaction of styrene oligomers with endocrine modulators (Gelbke et al., 2015; Ohyama et al., 2001). In this study, significant up-regulation of transcripts encoding the mussel type 1 (MeER1) and type 2 (MeER2) estrogen receptors and down-regulation of a transcript encoding a type 1 serotonin receptor (5-HT1) was observed in D-veligers grown in the presence of 10 μg/L styrene. The same transcripts were previously found to be modulated by BPA and by 17β-estradiol, leading to consider them as significant targets for estrogenic chemicals (Balbi et al., 2016).On the whole, these results indicate the potential of styrene to affect early development of the neuroendocrine system in mussels.

Interestingly, although in mammals styrene metabolism is known to generate ROS (reactive oxide species) as intermediate and final metabolites (Andersen et al., 2017), no significant modulation of transcripts encoding antioxidant enzymes was observed. As previously reported in mussel haemocytes exposed to pharmaceuticals (Franzellitti et al., 2016), this lack of toxicity may be ascribed to the protective function likely displayed by the MXR system that, by actively extruding styrene, may have lowered its oxidative potential.

5. Conclusion

The formation of styrene oligomers is considered a new and widespread source of hydrocarbon pollution leached from polystyrene marine litter (Kwon et al., 2014). In this light and considering the increasing amount of plastics discharged in the marine environment (Suaria et al., 2016), present data may raise concern over the potential threats represented by styrene-based compounds at environmental levels for the development of marine larvae. For example, Kwon et al. (2015) reported an average concentration of styrene oligomers of 0.5 μg/L in coastal waters of Italy, a value which falls within the LOEC to EC50 range assessed in this study through the embryotoxicity assay. In this same concentration range, morphological, functional (MXR activity) and transcriptional changes in styrene-treated embryos were also observed. We showed that main effects rely on transcripts underpinning physiological functions addressed as potential targets of styrene in adult mussels and in mammals, suggesting the possible conservation of styrene mode of action across bivalve life cycle and

between human and bivalves. Furthermore, unpredicted impacts on protective systems (i.e., alteration of the MXR-mediated active efflux) and on processes of shell biogenesis (i.e., altered gross shell morphology and impaired transcription of genes controlling calcium carbonate precipitation and shell growth patterning) were observed. Both processes are crucial for bivalve development (Balbi et al., 2016; Franzellitti et al., 2017). In particular, disrupting shell biogenesis performance at larval stages may forecast the onset of altered shell structural properties in adult stages, which are thought to be a main bottleneck determining vulnerability of bivalve populations towards environmental pollution and ongoing climate change (Gizzi et al., 2016; Ramesh et al., 2017).

Acknowledgments

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The Multixenobiotic resistance system as a possible protective response triggered by microplastic ingestion in Mediterranean mussels (*Mytilus galloprovincialis*): Larvae and adult stages

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ABSTRACT

The emerging paradigm on plastic pollution in marine environments is that microsize particles (MPs) have far more subtle effects than bigger fragments, given their size range overlapping with that of particles ingested by filter-feeders. The impacts include gut blockage, altered feeding and energy allocation, with knock-on effects on widespread physiological processes. This study investigated whether ingestion of polystyrene MPs (PS-MPs) triggers protective processes in marine mussels. The Multixenobiotic resistance (MXR) system is a cytoprotective mechanism acting as an active barrier against harmful xenobiotics and a route of metabolite detoxification. Both larvae and adults were employed in laboratory experiments with different concentrations of 3-μm PS-MPs (larvae), and 3-μm and 45-μm PS-MPs (adults) matching size range of planktonic food through the mussel lifecycle. Embryos grown in the presence of 3-μm PS-MPs showed significant reduction of MXR activity and down-regulation of ABCB and ABCC transcripts encoding the two main MXR-related transporters P-glycoprotein and the Multidrug resistance-related protein, respectively. In adults, effects of PS-MPs were assessed in haemocytes and gills, which showed different modulation of MXR activity and ABCB/ABCC expression according to MP size (haemocyte and gills) or particle concentration (haemocyte). These data showed that modulation of MXR activity is part of a generalized response triggered by particle ingestion.

1. Introduction

Microplastics, defined as plastic particles of size < 5 mm (UNEP, 2016), are ubiquitous in the marine environment and, depending on the size, they are readily ingested by all animals examined to date (Germanov et al., 2018; Lusher et al., 2017). In the most recent years, an increasing number of studies addressed the occurrence, distribution and bioavailability of MPs in the marine environment, showing the impacts of MPs on marine animals with adverse effects mainly related to altered digestive functions, decrease in food intake and tradeoff in allocation of energy sources (de Sá et al., 2018; Foley et al., 2018). Further effects were documented at the molecular and cellular endpoints related to oxidative stress, lysosome functions, immune-related functions, changes in metabolic pathways, neurotoxicity (Avio et al., 2015; Chen et al., 2017; Gaspar et al., 2018; LeMoine et al., 2018; PaulPont et al., 2018). Activation of cellular stress responses and the underlying signaling pathways was also reported (Jeong et al., 2017, 2016; Tang et al., 2018).

Being filter feeders, bivalve mussels (*Mytlius* sp.) inhabiting coastal habitats are prone to ingestion and accumulation of MPs, as they feed by collecting and sorting particulate matter to trap and ingest anything of appropriate size (Moore, 2008). These features make mussels ideal model species to investigate MP toxicokinetic in laboratory experiments with marine organisms (Paul-Pont et al., 2016; Van Cauwenberghe et al., 2015;

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Wright et al., 2013). The sensitivity of mussels to the physiological impacts of MPs have been also assessed, with transcriptomic and biomarker approaches setting the stage of investigations concerning sub-lethal effects of MPs (Avio et al., 2015; Bour et al.,2018; Capolupo et al., 2018; Magni et al., 2018).

This study attempts to infer the physiological impacts of MPs on the Mediterranean mussel (*Mytilus galloprovincialis*) by addressing a relevant cytoprotective mechanism that provide an adaptive advantage for mussels to cope with environmental challenges. Specifically, the study investigates modulation of the mussel Multixenobiotic Resistance System (MXR) in response to ingestion and accumulation of polystyrene MPs (PS-MPs). The MXR system endows marine mussels with an effective chemioresistance shield against a wide range of environmental stressors (Bard, 2000). The MXR is mediated by ATP-dependent ABC transporters, located on cell membranes and internal organelle membranes, which pump out from cells both endogenous chemicals and xenobiotics, thus preventing their accumulation and toxic effects (Bard, 2000). In marine bivalves, the best characterized ABC transporters are the P-glycoprotein (P-gp), encoded by the ABCB gene, and the Multidrug resistance-related proteins (Mrp) encoded by the ABCC gene (Franzellitti and Fabbri, 2006). Generally speaking, P-gp is considered a phase 0 transporter mediating the extrusion of unmetabolized compounds, while Mrp transporters constitute the phase III of biotransformation processes (Leslie et al., 2005). Besides their role in xenobiotic metabolism, induction of P-gp and Mrp in bivalves has been reported in response to a wide range of biotic and abiotic stressors, including algal toxins, pathogen infections, temperature or salinity variations (Buratti et al., 2013; Fu et al., 2019; Minier et al., 2000), suggesting that these transporters may be part of a general and broadspectrum cell protective mechanism that plays a vital role in acclimatization and response to environmental stress and the immune defense.

The possible effects caused by PS-MP ingestion were evaluated in terms of changes of total MXR efflux activity and transcriptional regulation of P-gp and Mrp in:

(i) Mussel embryos grown in the presence of different concentrations of 3 μm PS-MPs, whose effects on the MXR system were investigated at 24 h post fertilization (pf), when the first free swimming trocophorae larvae is developed, and at 48 h pf, when the fully developed D-shape shelled veliger is formed and advances in the feeding capabilities occurred (Dyachuk, 2016). At these embryo stages improvements of the MXR system were also observed (Franzellitti et al., 2017).

(ii) Gills and haemocytes from adult mussels (of commercial size) exposed in vivo to different concentrations of 3 μm and 45 μm PSMPs. These tissues have been selected as representing important active biological barriers in bivalves, and, as such, they are endowed with efficient protective mechanisms (Franzellitti et al., 2016; Luckenbach and Epel, 2008).

On the whole, this experimental approach build on the hypothesis that ingestion of MPs by mussels, a phenomenon observed in both adult and embryos life stages (Browne et al., 2008; Capolupo et al., 2018; Von Moos et al., 2012), may trigger the modulation of the MXR system as an accessory mechanism to protect the organism from the possible uptake of harmful agents (Whalen et al., 2010).

2. **Methods**

2.1. *Chemicals*

The ChargeSwitch total RNA cell kit was purchased from Thermo Fisher (Milan, Italy). The DirectZol kit was from Zymo Research (Freiburg, Germany). The iScript supermix and iTaq Universal master mix with ROX were from Biorad Laboratories (Milan, Italy).

Rhodamine123 (Rho123), the Tri-Reagent, and any other reagent was from Sigma Aldrich (Milan, Italy).

2.2. *Polystyrene microplastics (PS-MP)*

Fluorescently labeled polystyrene microspheres (PS-MPs) (Fluoresbrite Plain YG, 441/486 nm excitation/emission; 3 ± 0.15 μm and 45 ± 3.6 μm size diameter) were purchased from Polyscience Inc. (Washington, PA, USA). These MPs were selected as a proxy of MP exposure since their density (1.05 g/cm^3) is almost identical to that of artificial seawater (about 1.025–1.035 g/cm^3), so that they should not sink within 48 h from suspension in water. According to the manufacturer's specifications (Polyscience, Inc.), the PS-MPs are internally dyed using a dye entrapment technique and a highly hydrophobic YG dye, so that in aqueous environments it should remain trapped in the beads.Charge change is unlikely, as the microsphere are neutral and, according to the manufacture's specifications, only covalent interactions should be allowed to bind proteins or nucleic acids.

Sizes of MPs were selected to overlap size range of mussel planktonic preys (Van Cauwenberghe et al., 2015). Surfactants were not present or added to the original MP suspension whose medium was ultrapure water (Polyscience, Inc.). Stock solutions at the suitable concentrations were prepared in sterile distilled water, aliquoted and stored at 4 °C according to the manufacturer's specification. At each MP administration, 1 aliquot of the stock solution was serially diluted in sterile artificial seawater (ASW) to achieve the final treatment concentration in the microplate well (embryo treatments) or in the tanks (adult treatments). Before spiking, lack of aggregation was verified by epifluorescence microscopy.

2.3. *Animal handling*

Mediterranean mussels (*Mytilus galloprovincialis*) of commercial size (4–6 cm in length) were obtained from a government certified mussel farm (Cooperativa Copr.al.mo, Cesenatico, Italy). They were transferred to the laboratory in seawater tanks and acclimated in aquaria containing 35-psu filtered seawater at 16 °C with continuous aeration (> 90% oxygen saturation). During acclimatization, mussels were fed once a day with a commercial algal slurry (Koral, Xaqua).

2.4. *Larvae rearing and treatments*

A flowchart of the experimental setup is reported in Fig. S1A (Supplemental material). Gamete collection, oocyte fertilization and larvae handling were performed according to (Fabbri et al., 2014) and using spontaneously spawning mussels. Eggs were fertilized with an egg:sperm ratio 1:10 in 6-well (RNA extraction) or 12-well (MXR activity) cell culture plates. After 30 min, fertilization success (n. fertilized eggs / n. total eggs × 100) was verified microscopically (> 85%). At 30 min post fertilization (pf), 3 μm polystyrene microplastics (3 μm PSMP) were added to fertilized eggs in the proper wells from stock solutions suitably diluted to reach the final nominal concentrations of 50 and 500 particle/mL. Detailed treatment conditions are as reported by Capolupo et al. (2018). A control group (i.e. untreated embryos) was maintained in parallel to the experiment. Embryos were grown for 48 h in the presence of 3 μm PS-MP under constant temperature conditions (16 °C ± 1 °C). The 48-h embryotoxicity assay performed by Capolupo et al. (2018) under the same treatment conditions of this study showed no significant changes in the percentage of normally developed D-shape larvae between PS-MP treated samples and controls; therefore, samples at 24 h pf were embryos at the trocophorae stage, while embryos at 48 h pf were live D-shape shelled veligers.

2.5. In vivo exposure experiments with adult mussels

A flowchart of the experimental setup is reported in Fig. S1B (Supplemental material). A total of 180 mussels were randomly selected and divided in groups of 20 animals each and transferred to vessels containing 20 L of water. One liter of seawater per mussel is the suitable volume to avoid overloading and prevent the onset of unfavorable health conditions. For each experimental condition, 3 vessels (a total of 60 mussels) represented the 3 replicates. Mussels were treated for 4 days with 3 μm or 45 μm PS-MP at the nominal concentrations of 1 and 10 particle/mL. A group of unexposed (control) mussels were maintained in parallel to the treatment groups. Seawater was renewed each day and MPs added from concentrated stock solutions.

Haemolymph was extracted from the posterior adductor muscle of 12 individuals per vessel using a sterile 1-mL syringe and suitably pooled to obtain the total volume to perform each assay. Fresh haemolymph was immediately used for MXR activity assays or RNA extraction. After haemolymph withdrawal, gills were gently removed and washed in 35-psu sterile artificial seawater (ASW) to be processed for MXR activity assays or RNA extraction.

2.6. *MXR transport activity assays*

The assay was based on the analysis of cell-based transport using rhodamine 123 (Rho123), a fluorescent model substrate for both P-gp and Mrp transporters (Smital et al., 2000). Rho123 shows low to moderate rates of passive membrane permeation, so that it is effectively extruded by ABC efflux pumps (Luckenbach et al., 2014). Therefore, the amount of Rho123 in the cell is a measure of transporter activity: high activity of transporters is indicated by a weaker Rho123 fluorescence signal, whereas a stronger fluorescence signal corresponds to lower transporter activity.

Embryos were handled for MXR efflux assay according to Franzellitti et al. (2017). At each selected embryo stage, Rho123 (2.5 μM final concentration; stock solution prepared in DMSO, with solvent concentrations never exceeding 0.1% v/v) was added to treated and control wells to achieve a 90-min co-incubation period at 16 °C in the dark.

Haemolymph and gills were processed for MXR efflux assay according to Franzellitti et al. (2016). Freshly pooled haemolymph from each vessel was plated in 12-well plates (1 mL/well, 1 plate per vessel) and settled for 1 h at 16 °C in the dark. Cell attachment to the bottom of the well was checked by microscopic inspection. The medium was then removed, and after 2 washings, replaced with 1 mL ASW containing 2.5 μM Rho123 (stock solution prepared in DMSO; solvent concentration never exceeds 0.1% v/v). Several 6-mm diameter biopsies (biopsy punches, pfm medical, Köln, Germany) were taken from a single gill according to Della Torre et al. (2014), randomly arranged in 12-well plates (for each treatment: 4 biopsies from 4 different mussels within each well; one 12-well plate per vessel), and treated in 1 mL ASW using the same conditions employed with haemocyte treatments. For both haemocytes and gills, within each experimental trial, 12 wells from each 12-well plates were employed for each treatment level, which were randomly pooled to 4 technical replicates for each biological endpoint (N = 3).

The duration of exposure and Rho123 concentration were selected during preliminary experimental trials, which showed that these conditions did not significantly affect cell/embryo viability while providing the most stable and repeatable fluorescence readings (Franzellitti et al., 2017, 2016). At the end of the incubation period, samples have been processed according to Franzellitti et al. (2016) (gills and haemocytes from adults) and Franzellitti et al. (2017) (embryos). Fluorescence measurements were performed

using a Jasco FP-6200 fluorometer (λexcitation = 485 nm; λemission = 530 nm). Values were normalized to total protein content using the Qubit protein assay with the Qubit 2.0 system (Thermo Fisher) according to the manufacturer's instructions. Results were expressed as mean ± SEM of the percentage of variation relative to controls.

2.7. *Quantitative real-time PCR analysis of ABCB and ABCC mRNA expressions*

Total RNA extraction from mussel embryos was performed according to Balbi et al. (2016). Haemolymph was processed for total RNA extraction according to Franzellitti and Fabbri (2013). About 100 mg gill tissue was homogenized in 1 mL of the Tri Reagent and total RNA was further extracted using the DirectZol kit according to the manufacturer's protocol. In all samples, RNA concentration and quality were verified using the Qubit RNA assay (Thermo Fisher) and electrophoresis using a 1.2% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 600 ng (embryos) or 1 µg (tissue and cells) total RNA using the iScript supermix following the manufacture's protocol.

ABCB and ABCC mRNA expressions were assessed by quantitative real-time PCR (qPCR) using primer pairs and protocols reported previously (Franzellitti et al., 2017, 2016). A preliminary stability analysis of 6 established candidate reference transcripts was performed to achieve a robust normalization of qPCR data (Balbi et al., 2016; Franzellitti et al., 2015). The pairs helicase/elongation factor 1α and 18S rRNA/elongation factor 1α resulted the best performing reference gene products for qPCR data normalization with embryo and adult tissue samples, respectively. Calculations of relative expression of target mRNAs was performed by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne software tool (Thermo Fisher, Milan, Italy). Data were reported as mean ± SEM of relative expression (log_2 transformed fold changes) with respect to controls.

2.8. *Statistical analysis*

Data on Rho123 efflux assay were analysed using non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney U test, after deviations from parametric ANOVA assumptions being verified (Normality: Shapiro-Wilk's test; equal variance: F-test). These statistical analyses were performed using the GraphPad Prism6 software (GraphPad Inc.). Data from qPCR analyses were evaluated with the REST software (Pfaffl et al., 2002) that uses a randomisation test with a pairwise reallocation to assess the statistical significance of the differences in expression between each treatment-exposed group and the controls. Further comparisons between pair of treatments were performed using the Mann-Whitney U test. In all approaches, $P < 0.05$ was set as the threshold level of statistical significance.

Additional statistical approaches were used to evaluate:

(i) effects of different concentrations of 3 µm PS-MPs on MXR system across embryo development. Log-transformed variations of the target transcripts and log-transformed MXR efflux activities were used to calculate similarity matrices based on the Euclidean distance (999 permutations), and were submitted to permutation multivariate analysis of variance (PERMANOVA) using the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). Factors considered were “developmental stage” and “PS-MP treatment”. Pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance (Anderson et al., 2008). When the main test revealed statistical differences ($P < 0.05$), PERMANOVA pairwise comparisons were carried out. Distance-based redundancy

linear modeling (DISTLM) followed by a redundancy analysis (dbRDA) in PRIMER with PERMANOVA+ extension was also performed to examine the relationship between the multivariate dataset (i.e. the suite of transcripts assayed and their expression levels) and the predictor variables (life stage and PS-MP treatment). Numerical metric for life stage progression was indicated by the post fertilization time (0: unfertilized egg; 24 h: trocophora; 48 h: D-veliger). Treatment was indicated by the nominal concentrations of exposure (0, 50, and 500 particle/mL). DISTLM with a test of marginality was also performed to account for the contributions of ABCB and ABCC transcripts in explaining the total observed variance in the embryo PS-MP dataset. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria.

(ii) effects of different concentrations of 3 µm and 45 µm PS-MPs on MXR system in gills and haemocytes of adult mussels. Log-transformed variations of the target transcripts and log-transformed MXR activities were used to calculate similarity matrices based on the Euclidean distance (999 permutations), and were submitted to PERMANOVA using the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). Factors considered were "tissue", "size of PS-MPs", and "PS-MP treatment". Pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance (Anderson et al., 2008). DISTLM analysis followed by a dbRDA analysis in PRIMER with PERMANOVA+ extension was also performed to examine the relationship between the multivariate dataset (i.e. the suite of transcripts assayed and their expression levels) and the predictor variables (tissue type, size of PS-MPs, and PS-MP treatment). Numerical metric for tissue type was 1: gills, 2: haemocytes. Size of MPs was identified by the size diameter (3:3 µm size PS-MPs; 45:45 µm size PS- MPs). Treatment was indicated by the nominal concentrations of exposure (0, 1, and 10 particle/mL). DISTLM with a test of marginality was also performed to account for the contributions of ABCB and ABCC transcripts in explaining the total observed variance in the haemocyte and gill PS-MP datasets.

3. Results

3.1. *Effects of 3 µm PS-MPs on MXR efflux activity and ABCB/ABCC mRNA expressions in Mediterranean mussels across early developmental stages*

Mussel embryos were grown for 48 h in

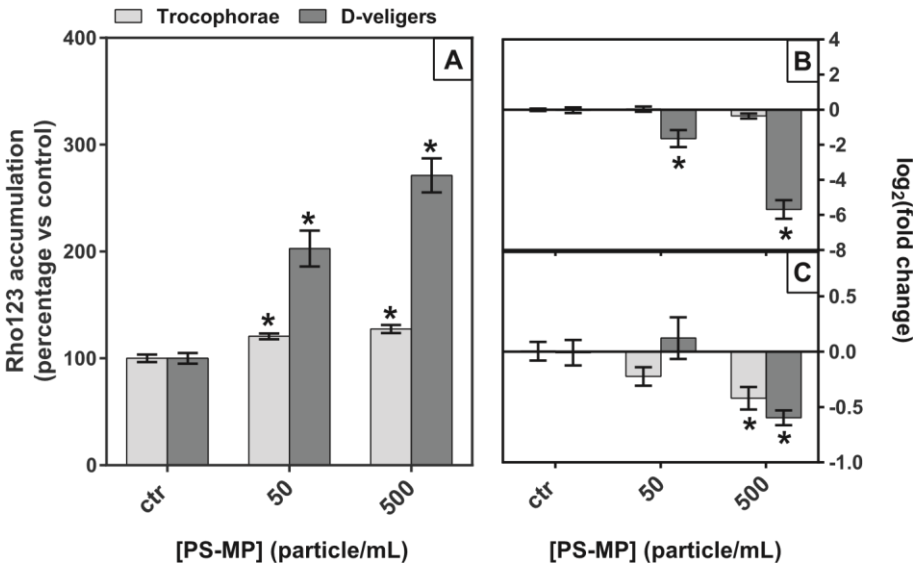

Fig. 1. Effects of 3 µm PS-MP ingestion on the MXR system in embryos of Mediterranean mussels across early developmental stages. (A) Rho123 accumulation. Data are expressed as mean ± SEM of 4 experiments carried out in 12-multiwell plates (3 replicate wells for each sample) (N =4) of the variation vs respective controls (ctr; untreated embryos). (B) ABCB (encoding the P-glycoprotein, Pgp) and (C) ABCC (encoding the Multidrug resistance related protein, Mrp) expression changes. Data are expressed as mean ± SEM of 4 experiments carried out in 6-multiwell plates (3 replicate wells for each sample) (N = 4) of the relative variation (log_2-normalized fold changes) vs respective controls (ctr; untreated embryo) within each stage (*P < 0.05).

the presence of 3 μm PS-MPs at 50 and 500 particle/mL. Compared to controls, Rho 123 accumulation was significantly increased by both PS-MPs treatments in all analysed embryo stages, indicating reduced MXR activities (Fig. 1A). In trocophorae, levels of the ABCB transcripts (encoding P-gp) were unchanged (Fig. 1B), while significant down-regulation of the ABCC transcript (encoding Mrp) was observed at 500 particle/mL (Fig. 1C). D-veligers showed significant ABCB down-regulation at both particle concentrations, while ABCC transcript was significantly down-regulated at 500 particle/mL.

Results from PERMANOVA demonstrated that both developmental stage and PS-MP concentrations significantly affected whole dataset and on the single biological endpoints ($P < 0.05$; Table S1), although in trocophorae permutation t-test analyses indicated no significant differences between either 50 particle/mL or 500 particle/mL PS-MP treatments and controls ($P > 0.05$; Table S2). A significant interaction between the two factors, developmental stage and PS-MP treatment, was observed on the whole dataset and on the single biological endpoints ($P < 0.05$; Table S1).

Distance-based linear model (DISTLM) analyses revealed that profiles of the biological endpoints were strongly dependent on the postfertilization time that explained about 76% total variation (Fig. 2A). Nevertheless, PS-MP treatment accounted for about 18.5% total variation explaining the observed changes in MXR-related parameters at the D-veliger stage (48 h pf) (Fig. 2A). DISTLM analysis by ABCB and ABCC transcript profiles showed that ABCB mostly explained the effects of PSMP in D-veligers, while a significant contribution of ABCC in both trocophare and D-veligers is observed (Fig. 2B).

3.2. *Effects of 3 μm and 45 μm PS-MP exposure on the MXR system in haemocytes and gills of adult Mediterranean mussels*

Adult mussels were exposed in vivo to 3 μm and 45 μm PS-MPs for 4 days at 1 and 10 particle/mL concentrations. Particle accumulation was assessed in haemolymph by epifluorescence microscopy (Fig. 3A, B). No MPs were observed in control samples, while particles were detected in haemolymph samples from all treated mussels with values being significantly different from controls in 3 μm PS-MP treated samples (1 and 10 particle/mL) and at 10 particle/mL 45 μm PS-MPs (Fig. 3C). PERMANOVA analysis reported in Table S3 showed that both particle size and nominal treatment concentrations significantly affected accumulation of PS-MPs ($P < 0.05$).

In haemocytes, reduction of Rho 123 accumulation was observed at 1 particle/mL concentration ($P < 0.05$), while a significant increasing is reported at 10 particle/mL (3 μm PS-MPs). Compared to control-exposed organisms, Rho123 accumulation was significantly reduced in haemocytes of mussels exposed to 45 μm PS-MPs, indicating increased total MXR activity (Fig. 4A). In gills, Rho123 accumulation was increased by 3 μm PS-MPs at 1 particle/mL concentration, while significantly reduced in gills from mussels exposed to 45 μm PS-MPs (10 particle/mL). ABCB showed general up-regulation compared to controls in both haemocytes and gills at all treatments (Fig. 4B). Expression of ABCC was significantly increased in haemocytes at both particle sizes and concentrations, except significant down-regulation is reported in samples exposed to 10 particles/mL 3 μm PS-MPs (Fig. 4B). In gills, ABCC expression was significantly down-regulated at both PSMPs sizes at 1 particle/mL concentration, while up-regulation and down-regulation was observed in the presence of 10 particle/mL 3 μm and 45 μm PS-MPs, respectively (Fig. 4C).

Results from PERMANOVA performed on whole haemocyte + gill dataset showed that tissue type, particle size and nominal treatment concentrations significantly affected the whole dataset and the single biological endpoints ($P < 0.05$), unless particle concentration did not significantly affect MXR efflux assay, and particle size did not significantly affect ABCC expression profiles (Table S4). Significant interactions amongst factors were also observed ($P < 0.05$; Table S4). DISTLM analyses performed on separate haemocyte and gill datasets are shown in Fig. 5A and B. In both tissues, profiles of the biological endpoints were dependent on particle size that explained about 73% total variation (Fig. 5A, B). Nevertheless, PS-MP nominal treatment concentration accounted for about 18% (haemocytes) and 15% (gills) total variation explaining the observed changes of MXR-related parameters, and a concentration related ordination with 3 µm and 45 µm PSMP treatments was observed in haemocytes (Fig. 5A). DISTLM analysis of ABCB and ABCC transcript profiles reported in Fig. 5C showed that only ABCC changes significantly contributed to explain the effects of PS-MP in both tissue datatsets and at both particle sizes.

4. Discussion

4.1. *The MXR response is down-regulated in mussel embryos exposed to PSMPs*

Our recent study (Capolupo et al., 2018) showed the ingestion of 3 µm PS-MPs by embryos of the Mediterranean mussel with increasing MP uptake across development progression and at increasing MP

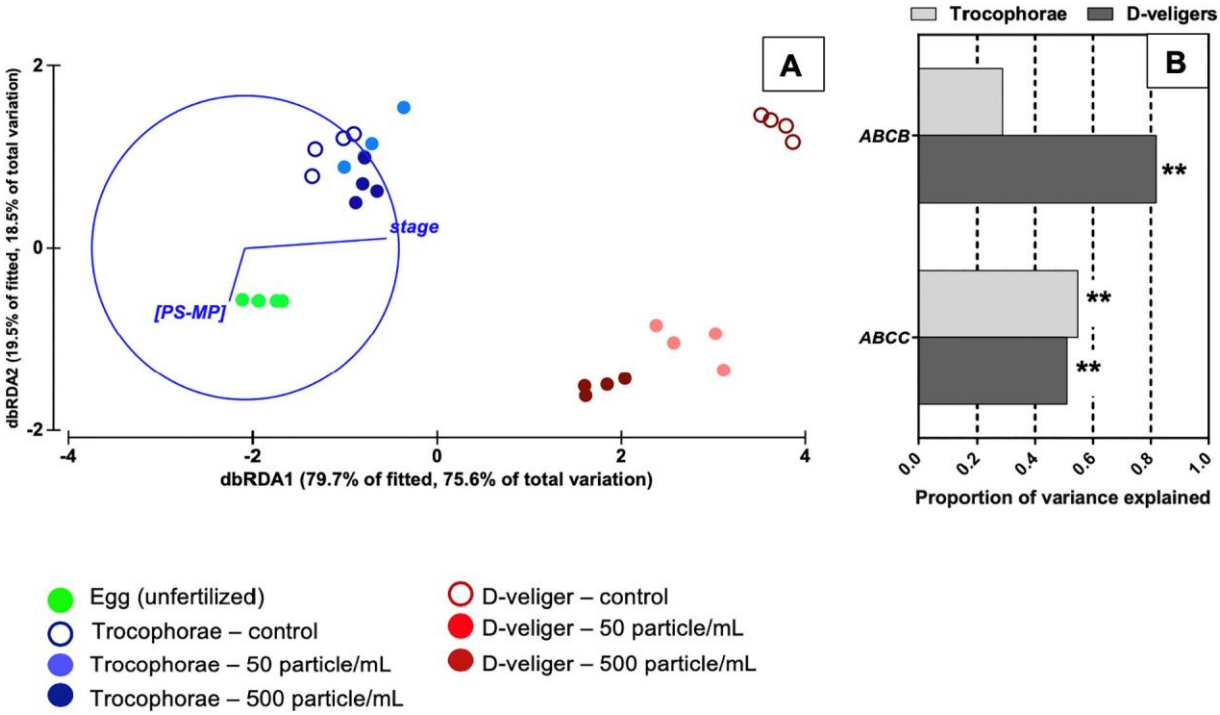

Fig. 2. Distance-based redundancy (DISTLM) modeling with distance-based redundancy analysis (dbRDA) to explore (A) the amount of variation in MXR activity and gene transcription to be attributed to PS-MP treatment of M. galloprovincialis embryos at different developmental stages. (B) DISTLM analysis showing contribution of ABCB and ABCC transcript profiles to the total variance observed in the dataset of trocophorae or D-veligers. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. Asterisks indicate level of statistical significance related to the result (Euclidean Distance resemblance matrix, 999 permutations; **P < 0.01; *P < 0.05).

concentrations. Furthermore, embryos showed prolonged MP retention (up to 192 h), suggesting an impact on digestive functions, while the 48 h embryotoxicity assay demonstrated that exposure to 3 µm PS-MPs did not significantly affect embryo development over a concentration range from 50 to 10,000 particle/mL (Capolupo et al., 2018). We further assessed the transcriptional regulation of several genes involved in different physiological processes across the 48-h pf period (Capolupo et al., 2018). Amongst these, we observed up-regulation of transcripts related to immune responses, and down-regulation of those involved in lysosome functions likely resulting from MP uptake and the consequent stimulation of the digestive and immune system, whose developments in bivalves are inextricably linked together (Balseiro et al., 2013; Dyachuk, 2016). Given the relevance of the MXR system for embryo physiology and its interactions with both immune and lysosomal functions, data reported in this study on MXR regulation attempt to integrate the general picture outlined by Capolupo et al. (2018) giving further hints into the physiological implications of MP interactions at the embryo stage.

Increased accumulation of the model fluorescent substrate Rho123 was observed in embryos treated with PS-MPs (trocophorae at 24 h pf, and D-veligers at 48 h pf), which indicate a reduction of total MXR efflux activity occurring under the experimental conditions that triggered the impairment of digestive functions and the molecular responses reported by Capolupo et al. (2018). Concomitantly, transcriptional modulation of ABC transporters was also observed. While trocophorae showed limited changes of efflux activity, no ABCB modulation and significant ABCC down-regulation, D-veligers displayed greater reduction of efflux activities and significant down-regulation of both ABCB and ABCC transcripts. Results on ABCB down-regulation are consistent with those obtained by Balbi et al. (2017) in mussel D-veligers grown in the presence of 150 µg/L 50-nm NH_2-polystyrene nanoparticles.

On the whole, these results pointed out two relevant findings. First, in both developmental stages observed reductions of efflux activity occurred together with transporter down-regulations (at least of Mrp/ ABCC). We recently showed that styrene monomer reduced MXR efflux activity in D-veligers without related transcriptional modulation of ABC transporters to occur (Rasika Wathsala et al., 2018). As in the case of styrene effects we hypothesized that the monomer could act as a MXR substrate rather than a modulator of ABCB/ABCC transcription, in this study the

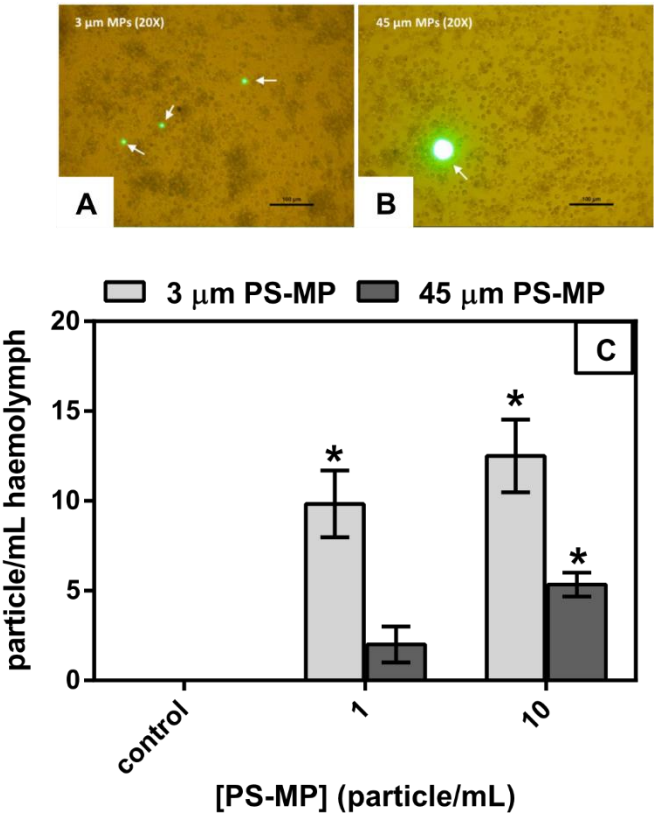

Fig. 3. Accumulation of 3 µm and 45 µm PS-MPs in haemolymph of adult Mediterranean mussels. (A, B) Representative images showing occurrence of MPs in hemolymph samples extracted from treated mussels. Images were acquired with an inverted epifluorescence microscope (Axiovert S100, Zeiss) equipped with a NIKON DS-Fi2 camera and the NIS-Element software (Nikon) (20× magnification; 365/397 nm excitation/emission filter). (C) The graph reports MP counts assessed in samples and normalized over the volume of hemolymph employed for each sample (mean ± SEM; N =6). *P < 0.05 vs control.

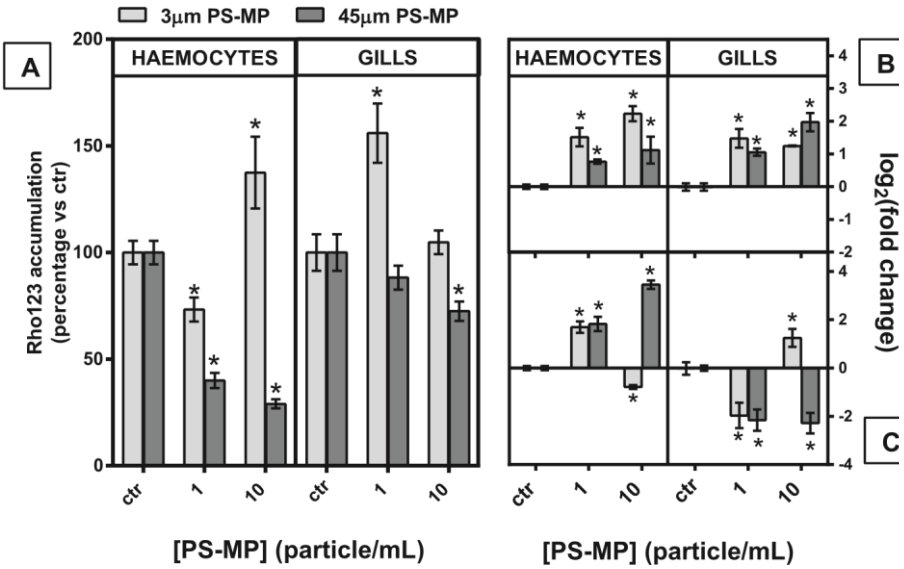

Fig. 4. Effects of 3 µm and 45 µm PS-MPs exposure on the MXR system in haemocytes and gills of adult Mediterranean mussels. (A) Rho123 accumulation in haemocytes and gills. Data are expressed as mean ± SEM of 3 experiments carried out in 12well plates (3 replicate wells for each sample) (N = 3) of the variation vs respective controls (ctr; untreated mussels). (B) Levels of ABCB (encoding the P-glycoprotein, P-gp) and (C) ABCC (encoding the Multidrug resistance related protein, Mrp) expressions in haemocytes and gills of mussels after a 4-days treatment with PS-MPs at the nominal concentrations of 1 and 10 particle/mL. Values (mean ± SEM) are expressed as the relative variations (log_2-normalized fold changes) between each treatment and control

coherent ABCB/ABCC regulation and total efflux activity suggest that ABC transporter down-regulations triggered by PS-MP exposure may be responsible of the observed changes in MXR efflux activity. The regulatory pathways determining this outcome are to be elucidated, and to date very few studies investigated the onset and modulation of cytoprotective responses towards MP exposure. For example, the monogonont rotifer Brachionus koreanus and the marine copepod Paracyclopina nana showed activation of antioxidant responses mediated by the MAPK/JNK pathway after ingestion of 0.05, 0.5 or 6 µm size PS-MPs (Jeong et al., 2017, 2016). Acute exposure to 1 µm PSMPs activated the stress response of the scleractinian coral Pocillopora damicornis concomitantly with induction of JNK and ERK signaling pathways (Tang et al., 2018). To this regard, it is worth noting that trocophorae and D-veligers grown in the presence of PS-MPs showed up-regulation of a transcript encoding a type 1 serotonin receptor (5HT1) (Capolupo et al., 2018). This may support the stimulation of the serotoninergic signaling by PS-MPs in embryos, in line with 5-HT1 upregulation previously reported in mussel haemocytes after in vitro treatment with the agonist serotonin (Franzellitti and Fabbri, 2013). Amongst other functions, serotonin is thought to modulate phagocytosis in invertebrates (Qi et al., 2016), and to regulate velum functionality, the embryo feeding organ (Braubach et al., 2006; Voronezhskaya et al., 2008). The increased serotoninergic signaling was observed to exert a negative control over ABCB transcription via negative regulation of the cAMP/PKA signaling (Franzellitti and Fabbri, 2013).

A further finding, highlighted by the DISTLM analysis, is that contribution of ABCB and ABCC transcriptional changes to the total variance observed in the dataset is dependent on the developmental stage analysed. Changes of total MXR activity in trocophorae appear mainly related to ABCC down-regulation, while in D-veligers modulation of both transporters seem to support the functional outcome. This is consistent with the reported ontogeny of the MXR system in mussels (Franzellitti et al., 2017), showing that MXR efflux in early embryos is mainly supported by the Mrp transporter while a significant P-gp mediated activity can be detected only from 48 h pf related to the increased demand of active protection against external stressors, and due to enhanced feeding. These data were paralleled by consistent ABCB and ABCC transcriptional profiles (Franzellitti et al., 2017). Data from Capolupo et al. (2018) showed that MP bioavailability is increased across development as larvae progressively improve their feeding capabilities. As such, the increased ABCB contribution to the total variance observed in the dataset may suggest

an enhanced interaction between embryos and PS-MPs.

Although both this study and the previous investigation (Capolupo et al., 2018) did not find any hints of overt physiological impairments by PS-MPs on mussel embryos, we may consider that MXR-related transporters, in particular Mrp, are thought to be integral to the developmental processes (Hamdoun et al., 2004). Thus, impairing the MXR system at early stages may be physiologically challenging for embryos, and the negative modulation exerted by ingestion of PS-MPs is likely to increase their vulnerability towards further environmental threats.

4.2. *The MXR response to PS-MP exposure in adults of M. galloprovincialis depends on tissue type and particle size*

The filter feeding behavior enables adults mussels to effectively ingest plastic particles of wide shape and size range, as demonstrated bylaboratory studies and the monitoring of natural and farmed populations (Bråte et al., 2018; Browne et al., 2008; Setälä et al., 2016; Van Cauwenberghe and Janssen, 2014). Gills represent a first site of MP uptake from the water, and are also sensitive to MP toxic effects (Magara et al., 2018). MP translocation towards different organs occurs via the circulatory system and is mediated by phagocytosis activity of the haemocytes (Browne et al., 2008; Kolandhasamy et al., 2018; Magni et al., 2018; Paul-Pont et al., 2016; Von Moos et al., 2012). Confocal microscopy demonstrated MP occurrence inside haemocytes within the circulatory system (Browne et al., 2008), and the formation of granulocytomas around accumulated polyethylene MPs was also observed in digestive glands of exposed mussels (Von Moos et al., 2012), showing that haemocytes are involved distribution of MPs bivalve tissues, and may be a target for MP effects.

On the whole, changes of MXR activity in gills and haemocytes resulted mainly dependent on MP size, since 3 μm PS-MPs mainly induced reduction of efflux activity, while increased efflux activity was observed in 45 μm PS-MP treated samples. This agrees with previous studies showing size-dependent outcomes of nanomaterials on different parameters assessed in mussels (Bouallegui et al., 2017; Katsumiti et al., 2016, 2015). Generally speaking, increased efflux activity is related to stimulation of the MXR system, while reduced efflux may derive from a negative transcriptional regulation of the transporters, or by a direct negative functional effect on transport activity (Leslie et al., 2005). Interestingly, increased haemolymph concentrations of the neurotransmitter dopamine was observed in freshwater zebra mussels Dreissena polymorpha exposed to 1 and 10 μm PS-MPs (Magni et al., 2018). The Authors postulated a possible implication of dopamine in mediating the elimination processes of accumulated MPs by increasing cilia movement in the gut epithelium and gills, in a manner similar to elimination of pseudofaeces. Therefore, a similar, and likely MP size dependent, signaling modulation due to the need for physiological control over gill and haemocyte functions, may have triggered the observed impairment of MXR system in the tissues. Amongst the possible involved neuromodulators, serotonin and noradrenaline are good candidates since they exert a strong control over bivalve gill and haemocyte functions (Carroll and Catapane, 2007; Dong et al., 2017; Lacoste et al., 2001b, 2001c, 2001a; Zhou et al., 2013). In mussels, both neuromodulators are believed to be involved in the transcriptional regulation at least of P-gp (Franzellitti et al., 2011; Franzellitti and Fabbri, 2013).

At the transcriptional level, DISTLM analysis showed that the main contribution to the total variance observed in both gill and haemocyte datasets is provided by ABCC

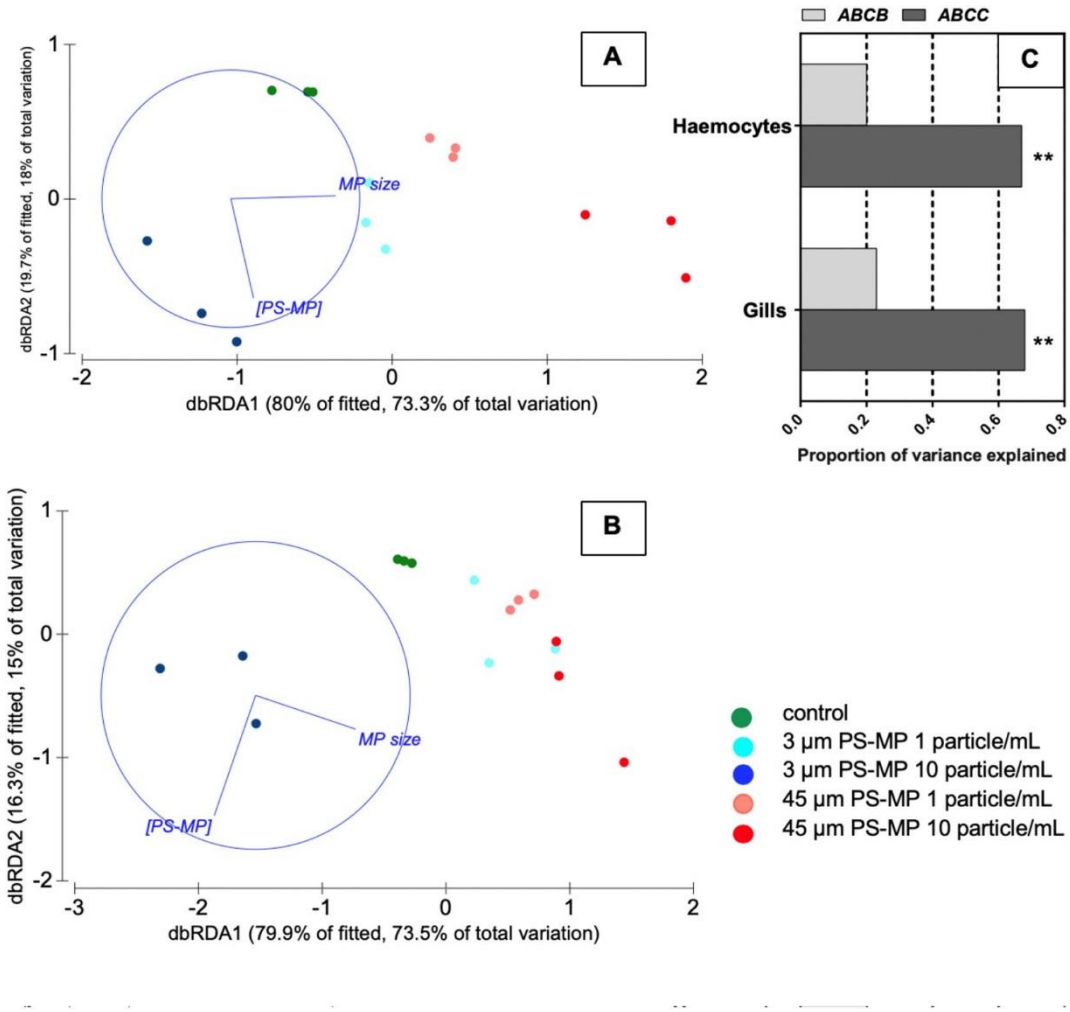

Fig. 5. DISTLM modeling with dbRDA analysis to explore the amount of variation in MXR activity and gene transcription to be attributed to PS-MP in vivo treatment of M. galloprovincialis haemocytes (A) and (B) gills at different developmental stages. (C) DISTLM analysis showing contribution of ABCB and ABCC transcript profiles to the total variance observed in the datasets. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. Asterisks indicate level of statistical significance related to the result (Euclidean Distance resemblance matrix, 999 permutations; **P < 0.01).

transcriptional changes, suggesting that variations of MXR efflux activity mainly rely on changes of the Mrp-mediated transport. Nevertheless, it is known that genes encoding ABC transporters undergo a complex regulation involving transcriptional, post-transcriptional (splicing, regulation of mRNA stability), and posttranslational phosphorylation, glycosylation, ubiquitination, proteasemediated degradation) processes likely operating with different temporal dynamics (Hennessy and Spiers, 2007; Scotto, 2003). Therefore, a (likely minor) contribution of the P-gp transporter cannot be excluded. Interestingly, although both ABCB and ABCC transcripts are involved in response to xenobiotics as well as to pathogen infections (Fu et al., 2019), ABCB has large differences in tissue expression patterns, as reported by an investigation on intra-specific and interspecific P-gp expression in clams (Huang et al., 2015). As to *M. galloprovincialis*, it is known that efflux activity in gills is supported by both P-gp and Mrp transporters, while in haemocytes a Mrp-dependent efflux prevails (Della Torre et al., 2014; Franzellitti et al., 2016). Furthermore, P-gp was induced under stress conditions, hence displaying a prevalent role in stress response (Franzellitti et al., 2016, 2010). On the whole, these findings lead to hypothesize that MP ingestion is likely to mostly interfere with physiological mechanism

of cell homeostasis (i.e. Mrp modulation) rather than inducing stress related effects (i.e. P-gp modulation).

Besides particle size, DISTLM and PERMANOVA analyses showed that in haemocytes the nominal concentration of PS-MPs was also a significant factor contributing to the functional outcome. Gills showed limited MP accumulation both under laboratory and natural exposure scenarios (Avio et al., 2015; Kolandhasamy et al., 2018; Von Mooset al., 2012), owing to their function as interface between the animal and the external environment. On the other hand, haemolymph is a site of MP accumulation (Browne et al., 2008), and results of this study further corroborate this finding, since accumulation of PS-MPs in the haemolymph of exposed mussels was observed by epifluorescence microscopy analysis. This may justify the hypothesis that cytoprotective responses in haemocytes are differently modulated in relation to the amount of accumulated MPs.

5. *Conclusions*

Data reported in this study suggest that modulation of the MXR system may be a part of a generalized response triggered by particle ingestion and stimulation of digestive and immune functions both in larval and adult stages of mussels (Capolupo et al., 2018; Paul-Pont et al., 2016). Constitutive and inducible expression of MXR-related transporters have been shown to serve as a protective counter-response in marine consumers by reducing dietary toxin absorption (Whalen et al., 2010), and to play a vital role in the immune defense (Fu et al., 2019). As such, the MXR system appears as an accessory molecular mechanism to be regulated alongside feeding. As for the embryos, a first hint into such a regulation related to MP exposure is reported in our previous study (Capolupo et al., 2018). In adults, future investigations should address this topic to disclose the extent to which specific tissue functions and the neuromodulatory control may affect the response to MPs, as already reported for other classes of emerging pollutants (Franzellitti et al., 2011).

Declaration of interest

None.

Transparency document

The Transparency document associated this article can be found, in online version.

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Appendix A. Supplementary figures and tables

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2019.02.005.

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Chapter 2

Studying basal expression of transcript involved in physiological processes of adult mussels under natural environmental gradients of physical/chemical parameters

In the second and third year, I worked on adult stages of *M. galloprovioncialis* to characterize in the broodstock the natural variability of the same molecular mechanisms assessed in larvae, under the influence of environmental gradients and gonadal development. To this aim physiological analyses were paralleled by investigations on microbiota composition at the tissue/organ level performed through Next generation sequencing approaches.

A preliminary **characterization of the structural variation of the microbiota of *M. galloprovincialis* at the tissue** scale was performed. Mussels were sampled during the spring season within a farm located in the North-Western Adriatic Sea, and microbiota composition was analyzed in gills, hemolymph, digestive glands, stomach, and foot by Next generation sequencing marker gene approach. Results allowed us to assess the distinctive microbiota structure of mussels compared to the surrounding seawater. Furthermore, each tissue was characterized by a distinct pattern of dominant families, reflecting a peculiar adaptation to the tissue-specific physiological activities.

A broader experiment was further performed to specifically address the impact of natural variability on both transcriptional profiles and microbiota composition. Gender bias was also considered. As the part of this study, we analyzed the stability of commonly used reference genes over different conditions: seasons, location, and gender to find the most stable reference genes for the quantification of target gene expression by qPCR in *M. galloprovincialis.*

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Tissue-scale microbiota of the Mediterranean mussel (*Mytilus Galloprovincialis)* and its relationship with the environment

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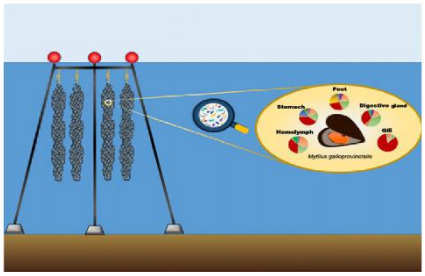
HIGHLIGHTS

- M. galloprovincialis possesses microbiota with specific declinations at the tissue level.
- The digestive gland microbiota is enriched in fibrolytic SCFA producers.
- Gill and hemolymph microbiota are dominated by aerobic marine microorganisms.
- By releasing gill microorganisms, mussel farms affect the surrounding water ecosystems.
- M. galloprovincialis microbiota play a role in different aspects of host physiology.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, we characterize the structural variation of the microbiota of Mytilus galloprovincialis at the tissue scale, also exploring the connection with the microbial ecosystem of the surrounding water. Mussels were sampled within a farm located in the North-Western Adriatic Sea and microbiota composition was analyzed in gills, hemolymph, digestive glands, stomach and foot by Next Generation Sequencing marker gene approach. Mussels showed a distinctive microbiota structure, with specific declinations at the tissue level. Indeed, each tissue is characterized by a distinct pattern of dominant families, reflecting a peculiar adaptation to the respective tissue niche. For instance, the microbiota of the digestive gland is characterized by Ruminococcaceae and Lachnospiraceae, being shaped to ferment complex polysaccharides of dietary origin into short-chain fatty acids, well matching the general asset of the animal gut microbiota. Conversely, the gill and hemolymph ecosystems are dominated by marine microorganisms with aerobic oxidative metabolism, consistent with the role played by these tissues as an interface with the external environment. Our findings highlight the putative importance of mussel microbiota for different aspects of host physiology, with ultimate repercussions on mussel health and productivity.

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1. Introduction

Mediterranean mussels (*Mytilus galloprovincialis*) Lamark,1819), like other bivalve mollusks, are key ecosystem engineers through their attachment to the substrate in dense mono- and multilayered beds. Alongside their ecological importance, mussels have a relevant economic value as a species of interest in aquaculture and, at the same time, have long been employed in the biomonitoring of environmental quality in coastal areas (Faggio et al., 2018; Capolupo et al., 2017; Carella et al., 2018; Moschino et al., 2016). Indeed, their powerful filter feeding activity links them to the surrounding environment, allowing to filter large volumes of water, while concentrating different types of waterborne or particulate pollutants as well as microorganisms (Gagné et al., 2019; Pagano et al., 2016; Neori et al., 2004).

Up to now, most of the microbiological studies on mussels focused on the identification of pathogenic bacteria with deleterious health effects (Erol et al., 2016; Richards et al., 2010; DaczkowskaKozon et al., 2010). However, marine organisms, including mussels, can be described as holobionts, given their life-long association with host-selected symbiont microbial communities known as microbiota (Pita et al., 2018; Glasl et al., 2016). These symbionts can endow the host with a set of probiotic functions (i.e. defense against pathogens, immunological regulation and improved nutritional efficiency), supporting homeostasis and health (O'Brien et al., 2019; Rausch et al., 2019; Simon et al., 2019). Consequently, the mussel microbiota should be considered as an integral component of the host physiology. However, to the best of our knowledge, only few and fragmentary studies aimed at the description of the mussel microbiota have been performed (Li et al., 2019; Vezzulli et al., 2018; Cappello et al., 2015; Kamada et al., 2013) and the resulting knowledge is still fragmentary.

The North-Western Adriatic Sea is characterized by a combination of shallow waters, restricted circulation and high riverine inputs (mainly through the Po river outflow) (Marini et al., 2008). These features affect its coastal areas that result as one of the most eutrophic environment and most productive area in the Mediterranean. Indeed, such conditions promote intense mussel farming, which is the prominent aquaculture activity in the area (Minarelli et al., 2018; Brigolin et al., 2017). Based on these considerations, mussel farms located in the North-Western Adriatic Sea are excellent field laboratories to explore the connection between the health and productivity of farmed mussels and the environmental quality. In an attempt to shed some light on the mussel microbiota structure and ecophysiology, here we applied a NextGeneration Sequencing (NGS) marker gene approach for the characterization of the symbiont microbial ecosystems of M. galloprovincialis collected from a mussel farm in the NorthWestern Adriatic Sea (Cesenatico, Italy). In particular, since recent studies carried out on the Manila clam (Ruditapes philippinarum) and the Pacific oyster (Crassostrea gigas) highlighted the existence of bivalve tissue-specific microbiota (Pathirana et al., 2019; Meisterhans et al., 2016; Lokmer et al., 2016), we explored the putative variation of M. galloprovincialis microbiota at the tissue scale. Hemolymph, gills, stomach and digestive gland are all important biological barriers between the animal and the environment, as well as sites for immunity, metabolism and detoxification (Freitas et al., 2019; Pagano et al., 2017; Franzellitti et al., 2016; Izagirre and Marigomez, 2009); hence, they host key functions for the mussel physiology. Thus, the dissection of

microbiome specific variations at these tissues can provide a comprehensive vision of the putative role of microbiota in the host physiology. Seawater samples collected around the mussel farm and 3 miles away were also analyzed for microbiota composition. By characterizing the symbiont microbiota of M. galloprovincialis at the tissue scale and its connection with the microbiota of surrounding water, we aim at providing the basic knowledge for further, applied studies, with the purpose of unravelling the role of microbiota in bivalve health and productivity, possibly in relation to anthropic pressure and environmental pollution.

2. Materials and methods

2.1. *Sampling and sample preparation*

Mussel (*M. galloprovincialis* Lam.) sampling was carried out in April 2019 (spring season) in a farm located in Cesenatico, Italy (position: 44°09′04″N 12°32′60″E), by professional fishermen of the "Cooperativa Promoittica" (Cesenatico, Italy). The location is approved for direct commercialization of mussels (European legislation 91–492-EEC) and it is sited within an area routinely monitored by the Regional Agency for Prevention, Environment and Energy of Emilia-Romagna, Italy (ARPAER) to assess the status of the marine ecosystem and seawater quality (https://www.arpae.it). Twenty-five mussels of commercial size (5–7 cm in length) were collected and immediately stored in coolers (+4 °C) to be transferred within a few hours to the laboratory. In the laboratory, the mussels were cleaned and gently washed and then dissected under sterile conditions.

Specifically, for each animal, hemolymph was taken from the posterior adductor muscle using a sterile 1-ml syringe and transferred to a sterile tube. A 100-µl aliquot was employed to assess the health status of the animals through the evaluation of lysosomal membrane stability (LMS) on mussel hemocyte cells, according to Buratti et al. (2013). LMS was employed in these preliminary assessments as it is a proven sensitive and reliable biomarker of general health status in bivalves (Viarengo et al., 2007). The digestive gland, foot, gill and stomach were dissected from each individual as well, snap-frozen in liquid nitrogen, and stored at −80 °C along with hemolymph until analysis.

Two liters of seawater were collected at a depth of 3 m near the mussel farm (position: 44°9′04″N 12°32′60″E), as well as 3 miles away from the collection site (44°5′53″N 12°35′28″E) (Fig. S1). Seawater samples were stored in coolers (+4 °C) during transport to the laboratory and then immediately processed. A summary of the samples, sample size and handling methods is reported in Table S1.

2.2. *Microbial DNA extraction*

Total microbial DNA was extracted from approximately 20–30 mg of the digestive gland, foot, gill and stomach, and from 200 µl of hemolymph, using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with only minor adjustments in the homogenization step. Specifically, all samples were homogenized using the FastPrep instrument (MP Biomedicals, Irvine, CA) at 6 movements per s for 1 min. The elution step was repeated twice in 50 µl, incubating the columns for 5 min at 4 °C before centrifugation. DNA samples were stored at −20 °C for subsequent processing.

Seawater samples were filtered on 0.45-µm pore size MF-Millipore membrane filters using a vacuum pump. Total microbial DNA was extracted from membrane filters using

the DNeasy PowerWater kit (Qiagen) according to the manufacturer's protocol.

2.3. *PCR amplification and sequencing*

The V3–V4 hypervariable region of the 16S rRNA gene was PCRamplified using the 341F and 785R primers with added Illumina adapter overhang sequences, as previously described in Barone et al., 2019. The PCR programused was as follows: 95 °C for 3 min as initial denaturation, then 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and 5 min at 72 °C for the final elongation. PCR reactions were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle PCR, using the Nextera technology (Illumina, San Diego, CA). After a further clean up step as described above, libraries were normalized to 4 nM and pooled. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 6 pM with a 20% PhiX control. Sequencing was performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions. Sequencing reads were deposited in SRANCBI (project number PRJNA604916, mussel samples from SAMN14002069 to SAMN14002182, seawater samples from SAMN14002225 to SAMN14002231).

2.4. *Bioinformatics and statistics*

Raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME 2 (Bolyen et al., 2019; https://qiime2.org). High-quality reads were clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using the VSEARCH classifier (Rognes et al., 2016) and the SILVA database as a reference (Quast et al., 2013). Unassigned sequences and those assigned to eukaryotes (i.e. chloroplasts and mitochondrial ones) were discarded.

Alpha rarefaction was performed using Faith's Phylogenetic Diversity (PD whole tree). A trade-off rarefaction value of 1900 reads per sample was chosen to capture the extent of diversity in our data. Beta diversity was estimated by computing weighted and unweighted UniFrac distances.

All statistical analysis was performed using R version 3.5.1 (https:// www.r-project.org/). Unweighted UniFrac distances were plotted using the vegan package, and the significance of data separation in the principal coordinates analysis (PCoA) was tested using a permutation test with pseudo-F ratios (function adonis in the vegan package). Alpha diversity was evaluated using two different metrics: Simpson Index (complement) and observed ASVs. Between-tissue differences for alpha diversity were assessed by Wilcoxon test. P-values were adjusted for multiple comparisons using the false discovery rate (FDR) (function p.adjust in the stats package), and a P-value $\leq$ .05 was considered as statistically significant. Representative sequences of taxa of interest were aligned to the 16S Microbial NCBI database (release September 2019) with BLASTn (version 2.9.0), considering at least 80% of sequences identity. Metagenome prediction of SILVA-picked ASVs was performed with PICRUSt2 (Barbera et al., 2019; Czech and Stamatakis, 2019; Douglas et al., 2019; Louca and Doebeli, 2018; Ye and Doak, 2009), using Metacyc (Caspi et al., 2018) as reference for pathway annotation and a NSTI threshold of 2. Over- abundant pathways in the different mussel organs and seawater were obtained in pairwise Wald tests, as implemented in DESeq2 package (Love et al., 2014). Over-abundant pathways with Bonferroni corrected P-value $\leq$.05 and an absolute ($\log_2$ fold change) $\geq$ 2 were retained. Sample clustering was performed accordingly to the pathways abundance profiles, adopting

Kendall's correlation coefficients as metric and Ward-linkage method.

3. Results

3.1. *NGS-based profiling of M. galloprovincialis microbiota and the surrounding seawater*

A total of 121 samples (25 digestive glands, 25 gills, 21 stomachs, 25 feet, 18 hemolymph samples and 7 seawater samples) were analyzed (Table S1). For each sample, the microbiota structure was profiled by NGS of the V3–V4 hypervariable region of the 16S rRNA gene. A total of 5,621,255 paired-end sequences passed quality filtering (mean per sample ± SD, 46456 ± 68,116). High-quality reads were clustered into 18,787 ASVs (8532 ± 4634).

The overall composition of the M. galloprovincialis microbiota is reported in Fig. 1A. The phyla Proteobacteria (mean relative abundance (r.a.) ± SD, 44.8% ± 27.2%), Firmicutes (18.5% ± 20.2%) and Bacteroidetes (14.8% ± 12.8%) dominated the ecosystem. Spirochaetes, Verrucomicrobia, Actinobacteria, Tenericutes, Planctomycetes, Cyanobacteria, Fusobacteria, Chloroflexi and Chlamydiae were subdominant components, with a mean r.a. of about 5%. At the family level, the most represented taxa were an unclassified family of the Alteromonadales order (10.7 ± 21.6%) and Flavobacteriaceae (8.8% ± 9.6%) (Fig. 1B). Spirochaetaceae, Ruminococcaceae, Lachnospiraceae, Bacillaceae, Vibrionaceae, Verrucomicrobiaceae, Hahellaceae and Rhodobacteraceae were subdominant families, showing a mean r.a. ranging from 3% to 5%. Consistently, among the dominant genera we reported unclassified taxa of Alteromonadales (10.6% ± 21.4%) and Flavobacteriaceae (5.4% ± 6.3%). Spirochaeta 2, Bacillus, Vibrio, Endozoicomonas, an unclassified genus of Verrucomicrobiaceae and Mycoplasma were all subdominant genera with mean r.a. between 3% and 5% (Fig. 1C).

As for seawater, Proteobacteria (68.6 ± 8.4%) and Bacteroidetes (14.8% ± 3.2%) were the dominant phyla (Fig. 1A), with Actinobacteria, Verrucomicrobia and Planctomycetes being subdominant components (mean r.a., 5%). The most represented families were Pseudoalteromonadaceae (11.6% ± 8.6%), Flavobacteriaceae (11.0% ± 4.3%) Vibrionaceae (9.3% ± 9.2%), Rhodobacteraceae (8.8% ± 4.0%) and Halieaceae (6.1% ± 5.9%). Microbacteriaceae, FamilyI of Cyanobacteria, Campylobacteraceae, Planctomycetaceae, and Verrucomicrobiaceae were subdominant components, with a mean r.a. ranging from 2% to 5% (Fig. 1B). At the genus level, Pseudoalteromonas (11.7% ± 8.4%), Vibrio (9.0% ± 8.7%), and unknown genera belonging to the Rhodobacteraceae (7.5% ± 4.8%) and Halieaceae families (6% ± 6.6%) were the dominant taxa. Among the subdominant ones, Synechococcus, Arcobacter and an unclassified genus of Verrucomicrobiaceae were present, all showing average r.a. between 2% and 3% (Fig. 1C).

3.2. *Tissue-specific composition of M. galloprovincialis microbial ecosystems*

To explore peculiarities of microbiota composition in the different tissues of M. galloprovincialis, an unweighted UniFrac-based PCoA of the compositional profiles of mussel samples, as well as of seawater, was carried out. As expected, the seawater samples clustered apart from all mussel organs (Fig. 2A) and the mussel samples significantly segregated according to the tissue type (permutation test with pseudo-F ratios, P-value ≤.001). To assess the degree of microbiota variation between tissues, pairwise adonis

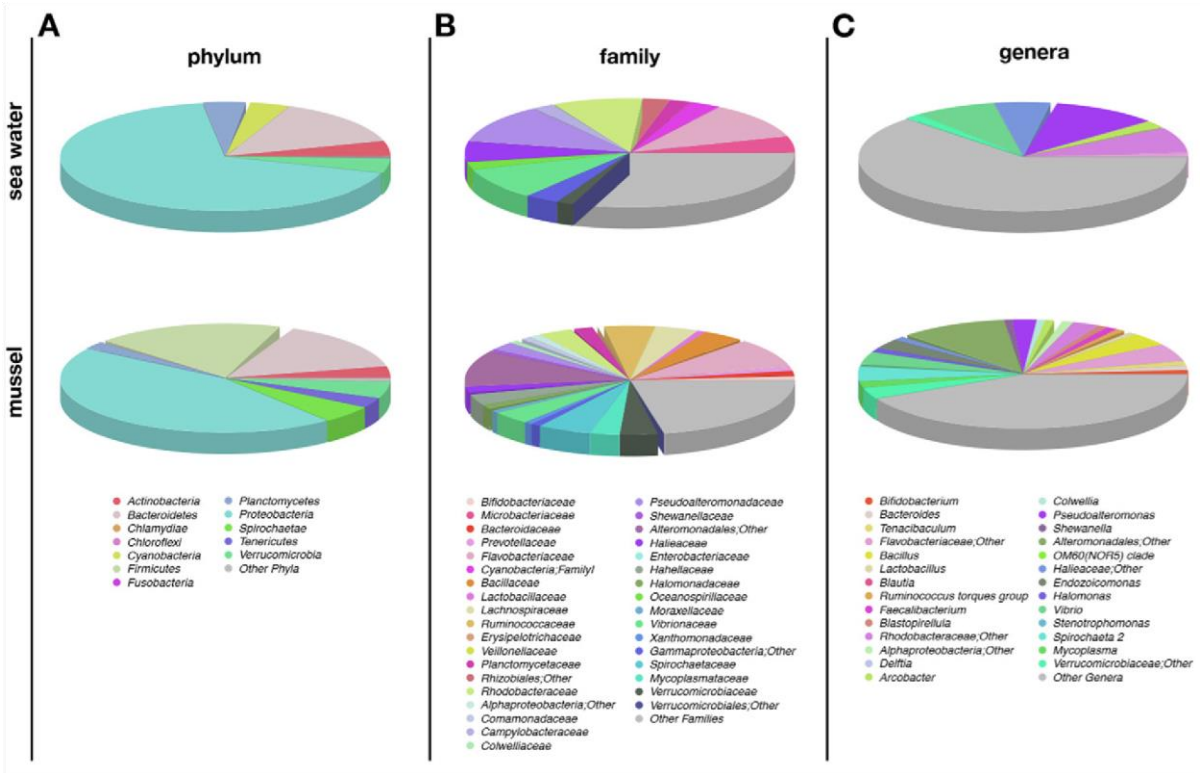

Fig1. The whole Mytilus galloprovincialis and seawater microbiota. Pie charts summarizing the phylum- (A), family- (B) and genus-level (C) microbiota composition of Mediterranean mussels and seawater. Only phyla with relative abundance ≥1% in at least 10% of samples, families with relative abundance ≥1.5% in at least 10% of samples and genera with relative relative abundance ≥2% in at least 10% of samples are represented.

permutation tests were performed (Table S2). Even if showing overall low R^2 values, all betweentissue comparisons of the microbiota structure were found to be significant (P-value ≤.03), highlighting the high level of organ specificity of mussel microbiota (Fig. 2A).

With regard to alpha diversity, no significant differences in species richness were found among the seawater and mussel ecosystems. However, the gill microbiota showed lower evenness (calculated as Simpson index-complement) than that of the digestive gland and stomach (Wilcoxon test, P-value b.03).

For what concerns the compositional structure, the microbiota from each organshowed a specific layout of dominant families (Fig. 3). In particular, Ruminococcaceae (mean r.a. ± SD, 14% ± 14%) and Lachnospiraceae (10% ± 13.2%) dominated the digestive gland microbial ecosystem. Spirochaetaceae were dominant in the foot (2% ± 26%), while an unclassified family of the Alteromonadales order (43% ± 25%) and Hahellaceae (11% ± 9.6%) dominated the gills, Mycoplasmataceae (15% ± 18%) the stomach and Flavobacteriaceae (19% ± 11.2%) the hemolymph (Fig. 3). The relative abundance of the most represented families in all Mediterranean mussel organs and seawater is provided in Table S3.

3.3. *Impact of mussel farming on the microbiota composition of the surrounding seawater*

Intending to assess the impact of mussel farming on the surrounding seawater, we compared the microbiota composition between 6 seawater samples collected close to the mussel farm and a sample collected 3 miles away from the farm as a control (Fig. S1).

As shown in Fig. S2, we noticed a variation in the family-level relative abundance profiles

between the seawater surrounding the mussel farm and the control water. In particular, the families Pseudoalteromonadaceae and Verrucomicrobiaceae showed greater relative abundance in the seawater surrounding the mussel farm than in the control water (mean r.a. ± SD, 13.4% ± 7.9% vs. 1%, and 1.9% ± 1.7% vs. 0%, respectively), while Halieaceae was more represented in the control water (r.a., 10.9% vs. seawater near the farm, 5.3% ± 6.4%).

Interestingly, the family Vibrionaceae, which includes several species known as opportunistic and potential pathogens of marine organisms (Baker-Austin et al., 2018; Le Roux et al., 2015), was also more represented in the seawater surrounding the mussel farm (10.6% ± 9.3%) than in the control water (1.5%). In order to identify the Vibrionaceaerelated ASVs down to species level, their sequences were mapped onto the 16S Microbial NCBI database. The best hit was Vibrio splendidus (N80% identity), a well-known potential pathogen.

3.4. *Predicted functional profiling of M. galloprovincialis and seawater microbiomes.*

To gain insight into the peculiar functional variations of the microbiota in the different M. galloprovincialis organs/tissues, as well as in the seawater, correspondent metagenomes were inferred from the phylogenetic profiles using PICRUSt2. A differential abundance analysis was carried out, resulting in 94 Metacyc pathways being significantly overabundant in at least one mussel organ or seawater metagenome (Supplementary Table S4). Samples were then clustered according to the abundance profile of the 94 over-abundant pathways (Fig. 4). Even if a certain level of dispersions was maintained, samples showed an overall tendency towards the segregation between water, gills and hemolymph. A cluster including stomach, digestive glands and foot samples was also obtained. The clustering analysis indicated for seawater a distinguished functional profile, characterized by the enrichment in pathways involved in nitrogen cycle (i.e. L-histidine degradation II and nitrate reduction VI) and in the degradation of the aromatic compound gallate. Although sharing several functionalities with the seawater microbiome, hemolymph was characterized by the over-abundance of pathways involved in sulfur metabolism (i.e. super-pathway of sulfolactate degradation), in the regulation of osmolarity (i.e. superpathway of taurine degradation and glycine betaine degradation I) and in the degradation of aromatic compounds (i.e. protocatechuate degradation II). Conversely, gills microbiome showed an enrichment in pathways involved in the respiratory electron transport (i.e. quinol and quinone biosynthesis). Notably, the digestive gland and the stomach microbiomes were both characterized by pathways involved in fermentation (i.e. pyruvate fermentation to acetate and lactate II and heterolactic fermentation) and in the degradation of several aromatic compounds (i.e. catechol, nicotinate, salicylate and toluene).

4. Discussion

In the present study, we characterized the Mediterranean mussel microbiota, also exploring its structural variation at the tissue scale and the connection with the microbial ecosystem of the surrounding seawater. According to our findings, the mussel microbiota was well differentiated from that of seawater. Indeed, at the phylum level the mussel microbiota was dominated by Proteobacteria, Firmicutes and Bacteroidetes, while that of seawater showed only Proteobacteria and Bacteroidetes as dominant phyla. However, it is at lower phylogenetic levels that the differences between the animal and seawater ecosystems were more evident. While showing a similar pattern of dominant

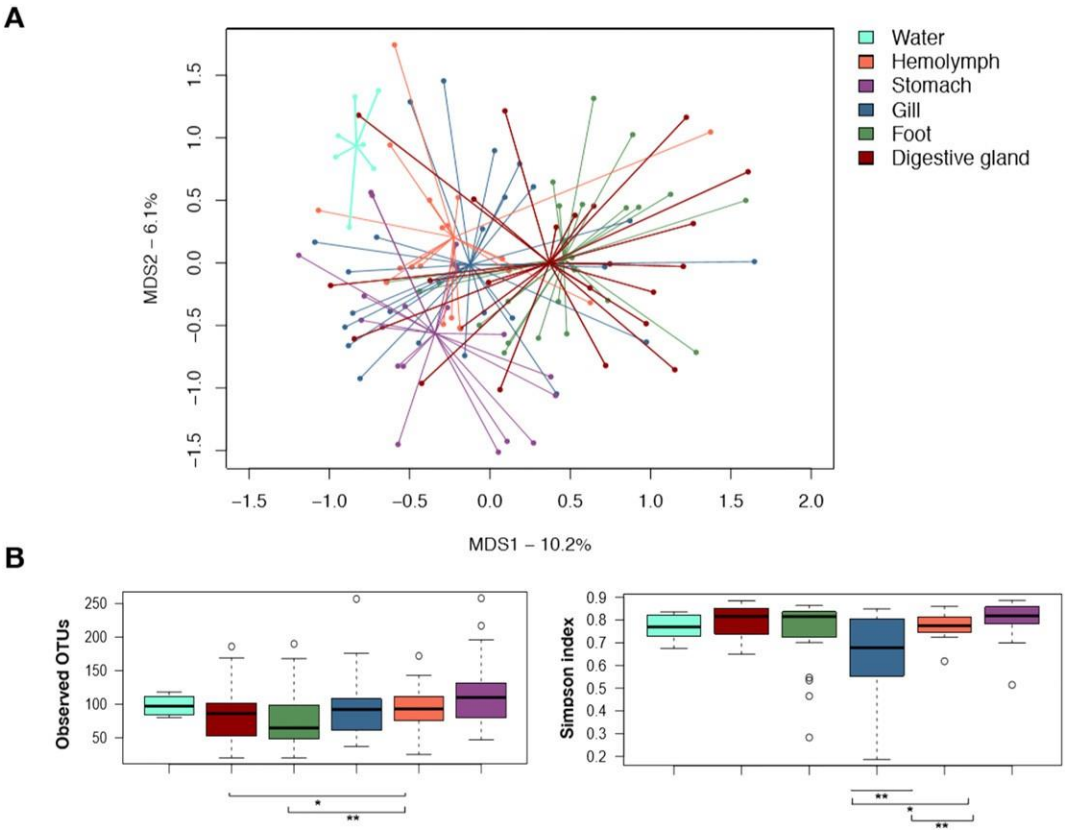

Fig. 2. Alpha and beta diversity of M. galloprovincialis tissue and seawater microbiota. (A) PCoA based on unweighted UniFrac distances between the microbiota structures of the samples taken from each organ of M. galloprovincialis and seawater. Samples are significantly separated (permutation test with pseudo-F ratios, P-value ≤.001). (B) Box and whiskers plots showing the alpha diversity values, measured as amplicon sequence variants (ASVs) and Simpson index- complement. *, P-value ≤.05, Wilcoxon test.

families, which mainly encompasses microorganisms of marine origin, such the plot in panel A. as Flavobacteriaceae, Alteromonadales and Rhodobacteriaceae, the subdominant fraction of the mussel and seawater ecosystems was remarkably different. Unlike seawater, which was characterized by a vast diversity of marine taxa, the mussel microbiota included well-known animal microbial commensals, such as Ruminococcaceae, Lachnospiraceae and Bacillaceae, and possibly opportunistic microorganism such as members of the family Spirochaetaceae. The inferred metagenomics also highlights an overall distinct functional profile between the seawater and the mussel microbiomes. While seawater was characterized by functions involved in sulfur and nitrogen cycling, the mussel ecosystems are enriched in genes involved in carbohydrates oxidation or fermentation, with specific variations depending on the tissue. Taken together, these data may indicate the propensity of mussels to select and retain microorganisms with animal tropism, such as symbiotic microbial partners. A similar behavior was recently reported for the sea cucumber, Holothuria glaberrima (Pagán-Jiménez et al., 2019), in which the presence of

Ruminococcaceae and Lachnospiraceae in the gut was suggested to influence the gastrointestinal metabolism of the host, as well demonstrated in terrestrial animals.

To better dissect the possible contribution of the mussel microbiota to the host physiology, we explored its variation at the tissue scale. Interestingly, although showing an overall comparable biodiversity, the microbiota of each tissue was characterized by a specific pattern of dominant families, suggesting a peculiar ecological propensity. For instance, being dominated by Ruminococcaceae and Lachnospiraceae, the digestive gland microbiota was configured as an anaerobic ecosystem enriched in commensal microorganisms capable of fermenting complex polysaccharides to short-chain fatty acids (SCFAs), well matching the general asset of animal gut microbiota (Muegge et al., 2011). Indeed, the digestive gland is the main site for the digestive, metabolic, and detoxification functions of mussels. These physiological activities may contribute to the establishment of suitable conditions to promote anaerobic bacteria capable of producing SCFAs through the fermentation of dietary fibers (Saltzman et al., 2017), such as cellulose and hemicellulose (La Reau et al., 2016), which are commonly found in bivalve food, as dinoflagellate algae (Arapov et al., 2010; Rouillon et al., 2005). Further supporting this considerations, a recent study showed that an α-D-glucan (MP-A) polysaccharide isolated from the mussel *Mytilus coruscus* affects the gut microbiota composition in Sprague Dawley rats fed with a high-fat diet, promoting SCFA production and

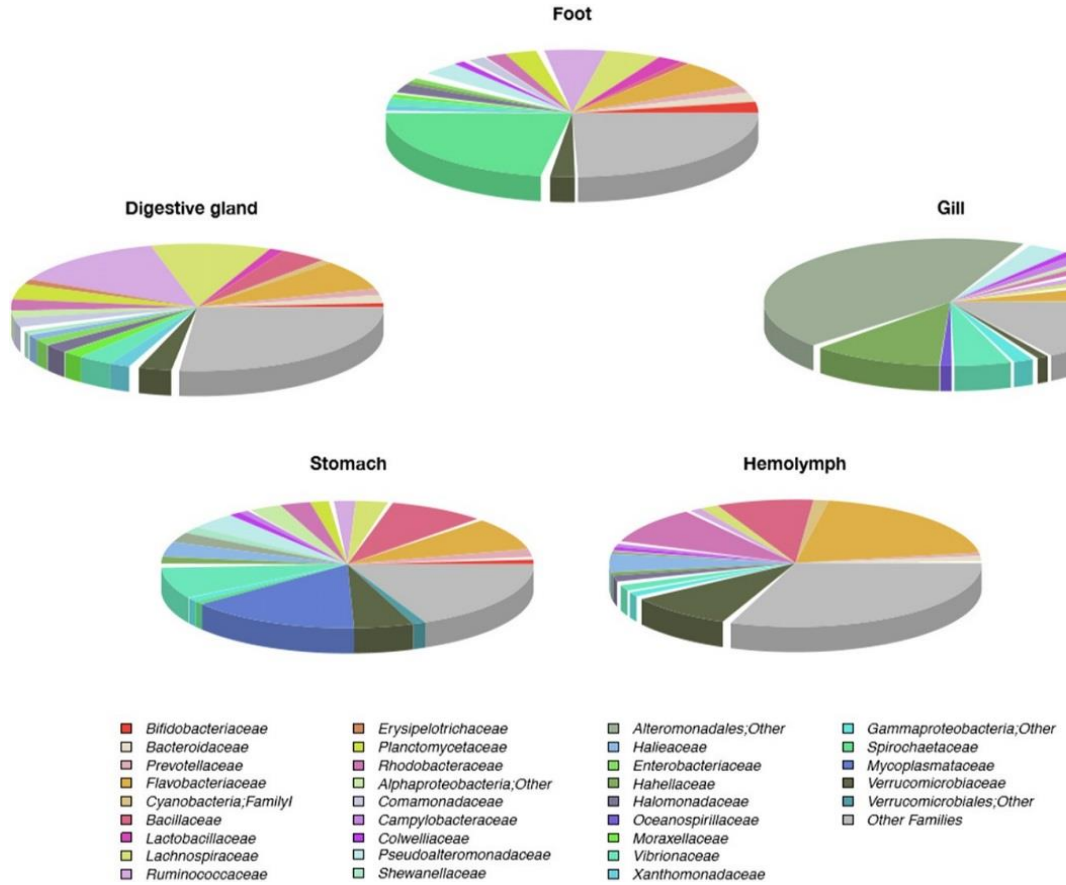

Fig. 3. The tissue-specific M. galloprovincialis microbiota composition at the family level. Pie charts summarizing the family-level microbiota composition of the digestive gland, foot, gill, stomach and hemolymph of M. galloprovincialis. Only bacterial families with a relative abundance of ≥1.5% in at least 10% of samples are represented.

alleviating the deleterious effects of the diet (Wu et al., 2019). Similarly, the stomach and foot microbiota were dominated by anaerobic microorganisms with animal tropism, especially Spirochaetaceae and Mycoplasmataceae. These microorganisms are generally considered as opportunistic rather than commensal, at least in mammalian hosts (Hampson and Ahmed, 2009; Waites and Talkington, 2004). However, according to van de Water et al. (2016), Spirochaetes members may act as symbionts in mollusks. Gills and hemolymph microbiota showed a completely different ecological structure compared to the other tissues, being dominated by aerobes of marine origin, such as Alteromonadales and Hahellaceae (gill), and Flavobacteriaceae (hemolymph). These findings well agree with the common role of the tissues as a primary biological barrier between the animal and the external environment, being in direct contact with the surrounding seawater. Therefore, their microbial composition most likely reflects the conditions imposed by the external environment, as observed in previous studies (Brito et al., 2018). On the other hand, gills and hemolymph may also exert an active role in the selection of microbial symbionts composing the microbiota of internal tissues (i.e. digestive gland and stomach), by means of filtering activity (gill), or immune recognition and phagocytosis operated by hemocytes and translocation to other organs/animal districts (hemolymph) (Ikuta et al., 2019; Burgos-Aceves and Faggio, 2017). Inferred metagenomes at the tissue scale confirmed the hypothesized metabolic propensities of the corresponding microbiota. Indeed, according to our findings, the gills ecosystem was characterized by functions involved in oxidative respiration, while the stomach and, particularly, the digestive gland, were over-abundant in functions related to the fermentation of polysaccharides and the degradation of aromatic compounds. These specificities of mussel microbiota at the tissue scale were robust to inter-individual variability. This suggests that the main determinant of the mussel microbiota variation is the niche-specificity rather than the individual differences. The same behavior was observed for mammals, where the structure of symbiont microbial ecosystems segregates according to the body district (Integrative HMP-iHMP- Research Network Consortium, 2014).

Finally, we explored the impact of mussel farming on the microbiota of the surrounding water. Compared to the control seawater (i.e. water collected 3 miles away from the mussel farm), seawater collected close to the farm was enriched in Pseudoalteromonadaceae, Verrucomicrobiaceae and Vibrionaceae, while being depleted in Halieaceae. This data emphasizes the potential of mussel farming to directly affect microbial ecology of seawater by releasing microorganisms that characterize the gill (i.e. Vibrionaceae and Pseudoalteromonadaceae) and hemolymph (i.e. Verrucomicrobiaceae), while retaining Halieaceae in the mussel microbiota. These findings further stress the close contact between gill/hemolymph and the external environment, as well as the function displayed by both tissues as the main route for tissue uptake of waterborne compounds and particulate material (including microorganisms).

5. Conclusions

Our study provides the first integrative description of the mussel microbiota variation at the tissue scale. According to our findings, mussels possess a characteristic microbiota, well differentiated from the seawater micro-ecosystem, with robust compositional variations at the organ level. Indeed, while gill

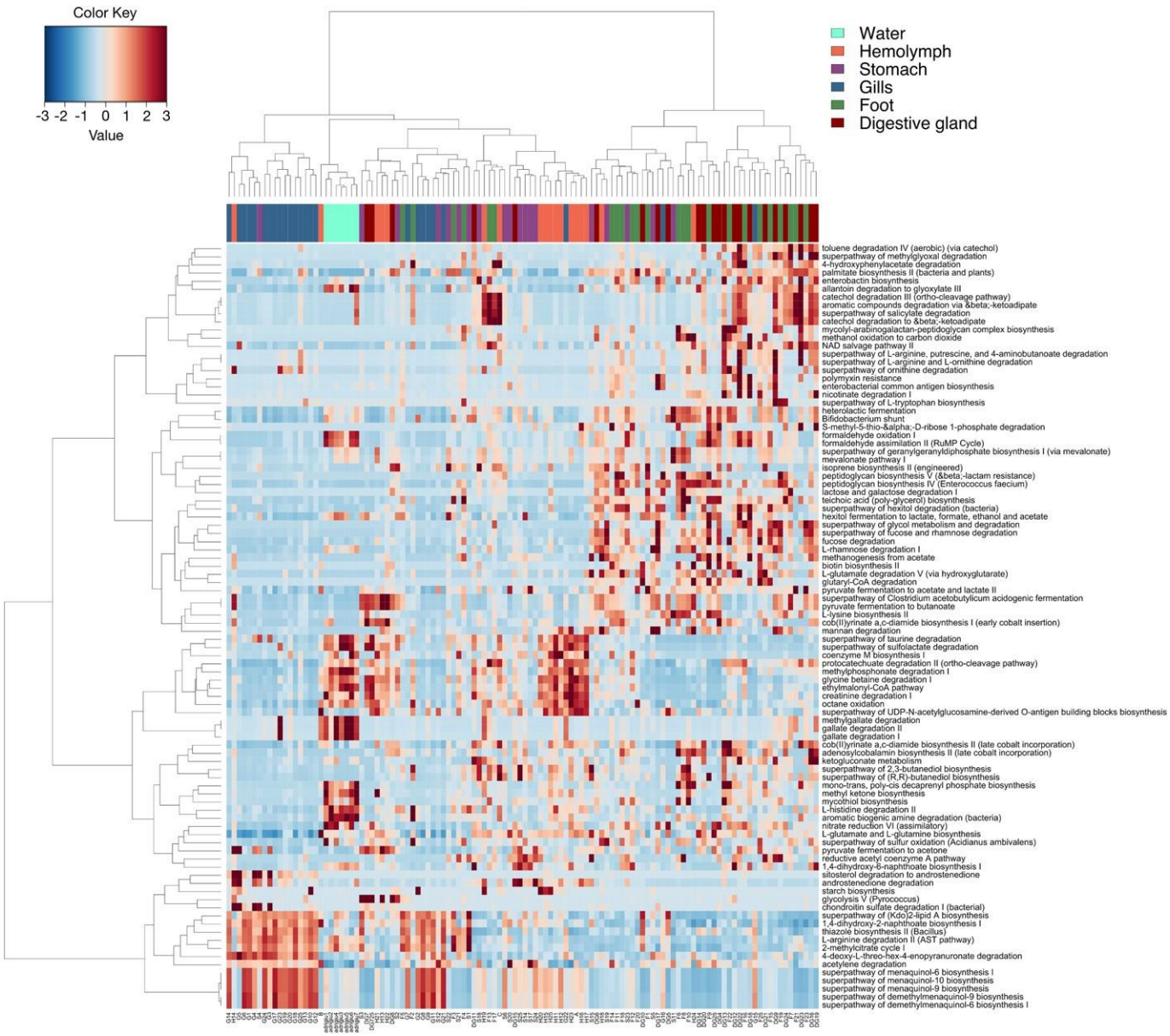

Fig. 4. Hierarchical clustering of the inferred metagenomes from the different tissue of M. galloprovincialis and seawater. The heatmap shows Ward-linkage clustering based on the Kendall correlation coefficients of the sample abundances profile of the 94 over-abundant pathways (Wald test logarithmic fold change of 2, P-value ≤.05). Samples are shown column-wise and colored by tissues. Metabolic pathways, named from the Metacyc database, are reported on the rows.

and hemolymph ecosystems are generally dominated by aerobic marine microorganisms, foot, stomach and digestive gland microbiota are characterized by anaerobes with tropism for animal tissues. In particular, being dominated by Ruminococcaceae and Lachnospiraceae, the microbiota of the digestive gland appears to be well structured for the production of SCFAs from complex polysaccharides. As health-promoting bioactive small molecules (Turroni et al., 2017), SCFAs may be effective in modulating the host metabolic and immunological layout (Hu et al., 2018; Ikeda-Ohtsubo et al., 2018), providing the mussel holobiont with important probiotic functions, including immune stimulation and cell signaling. Our findings promote further research to better understand

the role of mussel microbiota in different aspects of host physiology. In particular, by means of shotgun metagenomics more information could be provided on the mussel microbiome structure also in response to environmental and anthropic stressors, to highlight the ultimate impact on health and productivity. Likewise, the temporal dynamics of mussel microbiota in the different organs need to be described, to dissect the key time windows and developmental stages for the microbiota establishment.

Supplementary data to this article can be found online at **https://doi.org/10.1016/j.scitotenv.2020.137209**

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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. **Supplementary data 4**

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Variability of metabolic, protective, and antioxidant gene transcriptional profiles and microbiota composition of Mediterranean mussels (*Mytilus galloprovincialis*) farmed in the North Adriatic Sea (Italy)

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1. INTRODUCTION

The marine ecosystem is interconnected with the terrestrial ecosystem, and thus, changing any component in each system automatically affects another's functioning. In particular, coastal habitats are severely impacted by the contaminants receiving from the terrestrial surroundings that affect organism health, biodiversity, and consequently, ecosystem functioning (Islam and Tanaka, 2004; Lacroix et al., 2017). Besides chemical pollution, changes may also occur due to climate changes such as seawater warming, salinity variations, ocean acidification (Gruber, 2011). In this context, investigations of the regulatory mechanisms governing stress responses of marine organisms may elucidate on the critical pathways setting the limits of animal acclimatization to anthropogenically modified marine environments.

Marine mussels (*Mytilus* spp.) dominate sessile fauna of many coastal areas and estuaries. When considering adult mussels as the keystone species residing in the coastal habitat, their sessile nature and filter-feeding behavior lead to chronic exposure to pollutants (Fernández et al., 2010; Petes et al., 2008).

Environmental studies with mussels highlighted seasonal fluctuation of microbial indices, contaminant bioaccumulation, cellular biomarkers, and key physiological functions, including spawning (Azizi et al., 2018; Caricato et al., 2010; Ivanković et al., 2005; Roméo et al., 2003; Sheehan and Power, 1999; Shen et al., 2020; Vernocchi et al., 2007), and suggest the influence of abiotic factors (temperature, pH, salinity, food availability), and biotic

factors, like gender bias and reproductive stage, for those biological responses (Blanco-Rayón et al., 2020; Grbin et al., 2019; Grenier et al., 2020).

Molecular biomarkers are powerful tools to infer mussels physiology, but only a few studies emphasized that gene expression is modulated by natural variation (Banni et al., 2011; Counihan et al., 2019; Schmidt et al., 2013). However, limited knowledge on mussel regulatory mechanisms is available, making it difficult to distinguish their physiological responses as natural responses or stress-induced responses, thus leading to misinterpretation of field data.

This study evaluates the transcriptional profiles of genes related to metabolic, detoxification, antioxidant and lysosomal responses in Mediterranean mussels (*Mytilus galloprovincialis*) under the influence of natural environmental seasonal gradients of environmental variables, gender bias, and gonadal development. We attempt to identify those environmental parameters and/or endogenous factors that may modulate some functional categories of stress-responsive gene transcripts, likely affecting the capability of the animals to cope with further environmental changes or the occurrence of natural and anthropogenic toxins. Furthermore, a recent literature review (Lindsay et al., 2020) shows that the composition of gut microbial community of a species can vary seasonally with host diet, metabolic demands, and life stage. These changes seem to comprehensively contribute to the host flexibility to cope with environmental changes, enabling the host to live within different environments, adapt to seasonal changes and maintain its physiological performances. Therefore, this study further explores basal responses of the mussel digestive gland (DG) microbiome to seasonal changes, allowing to figure out acclimatory microbiome variations likely matching host physiological changes across seasonality.

2. METHODS

2.1. Mussel sampling

Five sampling campaigns were performed from a mussel farm located in the northern Adriatic Sea by professional fishermen of the "Cooperativa Pro.mo.ittica" (Cesenatico, Italy). The selected sampling site (Fig 1A) is located within an area routinely monitored by the Regional Agency for Prevention, Environment and Energy of Emilia-Romagna, Italy (ARPA-ER) to evaluate the status of the marine ecosystem and seawater quality parameters. Mussel samples were collected once a month, from February to August 2018. At each sampling time point, 60 randomly selected mussels were immediately stored in coolers (+4°C) and transferred to the laboratory, where they were cleaned and washed, and then dissected under sterile conditions to collect mantle/gonad complexes and digestive glands for molecular analyses.

Temporal trends of seawater parameters (transparency, temperature, surface oxygen, salinity, pH, and chlorophyll-a) were retrieved from the web portal of the Regional Agency of Environmental Protection, ARPA (https://www.arpae.it) (Fig 1B).

Sex was determined in individual mussel using the sex-specific gene method (Fraser et al., 2016). Specifically, the method consists in the quantification through quantitative real-time PCR (qPCR) assays of expression of the mussel vitelline envelope receptor for lysine (*VERL*), and vitelline coat lysine (*VCL*) mRNAs in the mantle/gonad complex. The transcripts are specifically expressed in females and males, respectively (Hines et al., 2007; Sedik et al., 2010). This method proved suitable differentiating males from females both during gametogenesis and sexual resting stage, when histology does not allow the observation of gametes (Fraser et al., 2016). RNA extraction and cDNA preparation from mussel mantle/gonad complex was as reported below. qPCR reactions were performed in

duplicate for each mantle/gonad sample using primer pairs and protocols reported previously (Anantharaman and Craft, 2012) (**Table S1, See supplement 5**). Threshold cycle (C_T) values were determined by setting a constant baseline and using the Expression Suite software ver 1.3 (Thermo Scientific). Sex was determined calculating the intra animal ΔC_T as C_T(VCL) – C_T(VERL) (Anantharaman and Craft, 2012); negative values indicate males and positive values indicate females. Relative VCL or VERL expression values across season (Fig 1C) were inferred by a comparative C_T method (Schmittgen and Livak, 2008) using the normalization and statistical strategy reported below.

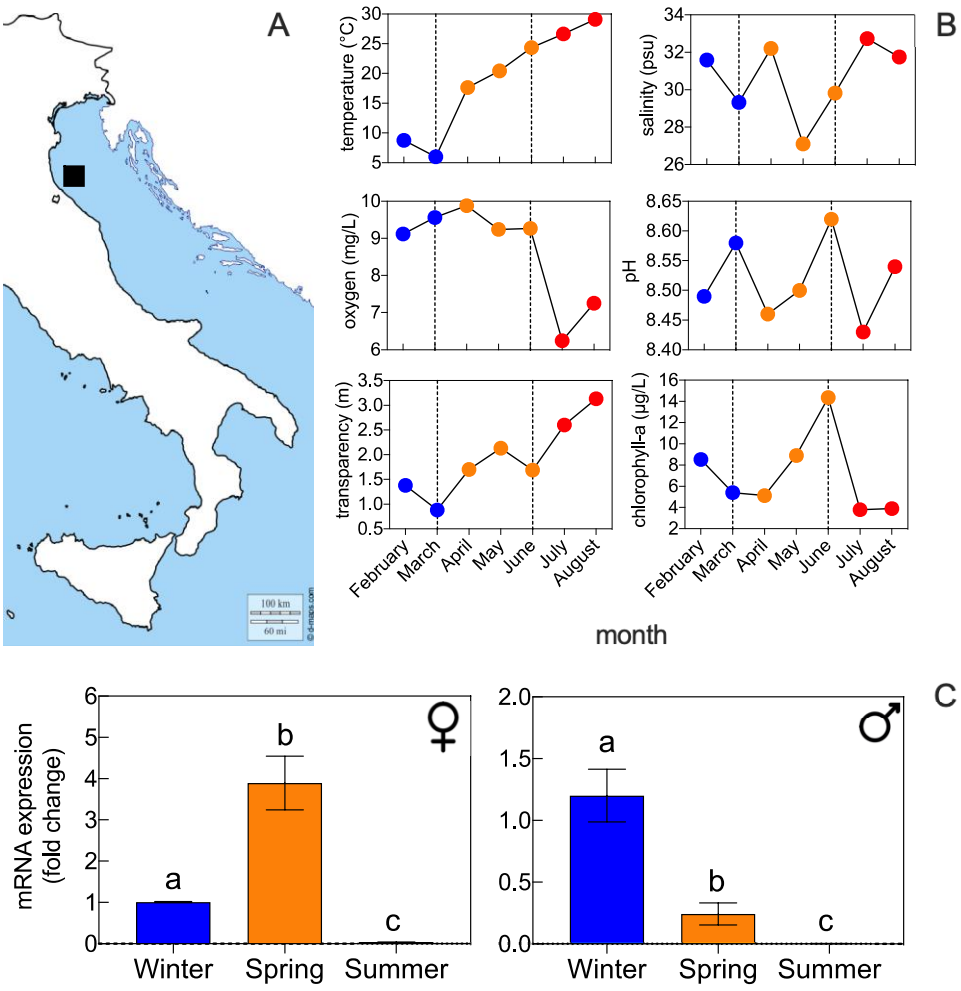


Fig 1. Study area, variations of environmental parameters, and gonadal cycle across the sampling period. (A) Location of the mussel farm assessed in this study in the North-West

Adriatic Sea (Italy). Map was generated using the Dmaps database (https://d-maps.com). **(B)** Temporal trends of sea water parameters at the sampling location. Data are retrieved from the web portal of the Regional Agency of Environmental Protection, ARPA (https://www.arpae.it). Blu dots: winter; orange dots: spring; red dots: summer. **(C)** Transcriptional profiles of male-specific vitelline coat lysin (*VCL*) and female-specific vitelline envelope receptor for lysin (*VERL*) mRNAs in mantle/gonads of farmed Mediterranean mussels addressing trends of gonad maturation across seasons.

2.2. RNA extraction, cDNA preparation, and qPCR analyses

For each animal, mantles (sex identification and gonadal cycle) and digestive glands (200 mg of tissue) were independently homogenized in a suitable volume of the TRI Reagent (Sigma Aldrich, Milan, Italy) and total RNA was extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. RNA concentration and quality were confirmed using the Qubit system with the Qubit RNA assay kit (Thermo Scientific, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. The analysis of UV absorbance spectra of the samples (λ = 200 – 340 nm) allowed the calculation of Absorbance (A) ratio A260/A280 addressing the occurrence of protein contaminations (cut-off values > 1.8 and < 2.0), and the ratio A260/A230 addressing the occurrence of contaminants that may be present in the samples, such as guanidine thiocyanate, which is a component of the TRI Reagent (cut-off value > 1.7). First strand cDNA for each sample was synthesized from 1 µg total RNA using the iScript supermix (BioRad Laboratories, Milan, Italy) following the manufacturer's instructions.

Expression profiles of selected transcripts in digestive glands were assessed by qPCR using primer pairs listed in **Table S1 (Supplement 5)** and protocols reported in previous studies (see references in Table 1). 18S and 28S were selected as reference gene products for qPCR data normalization by a preliminary stability analysis of 6 established candidate transcripts (Balbi et al., 2016). Relative expression values of target mRNAs were inferred by a comparative C_T method (Schmittgen and Livak, 2008) using the StepOne and DataAssist

softwares (Thermo Fisher, Milan, Italy). Data were reported as relative expression (fold change) with respect to a reference sample (Winter male).

2.5. Microbial DNA extraction and sequencing

Total microbial DNA was extracted from approximately 20 – 30 mg of digestive gland tissue using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to (Musella et al., 2020). The V3–V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 785R primers with added Illumina adapter overhang sequences, as previously described (Barone et al., 2018). The thermal cycle consisted of initial denaturation at 95°C for 3 minutes, 30 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and 5 minutes at 72°C for final extension. PCR reactions were then cleaned up with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle PCR, using the Nextera technology and then pooled after a further clean up step as described above and normalized to 4 nM. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 6 pM with a 20% PhiX control. Sequencing was performed on Illumina MiSeq platform using a 2 × 250 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). Sequencing reads were deposited in SRA-NCBI (*project Accession Numbers will be available upon manuscript acceptance*).

2.6. Statistical and bioinformatic analyses

qPCR data were analyzed using the REST software (Pfaffl et al., 2002) to test for statistical differences in mRNA levels of the treatment groups vs the reference condition. Further pairwise comparisons were performed with the Mann-Whitney U test. Correlation analyses (Spearman's test), data visualization, and graphics were obtained with the ggplot2 R

package in R (R Development Core Team, 2018). In any case, statistical differences were accepted when $P < 0.05$.

The complete dataset was further analyzed by a 2-way permutation multivariate analysis of variance (PERMANOVA) using PRIMER v6 (Anderson et al., 2008) to test for variations of transcriptional profiles amongst sex and season groups. Log-transformed variations of the target transcripts were used to calculate similarity matrices based on the Euclidean distance (999 permutations; P(perm) < 0.05). When the main tests revealed statistical differences ($P < 0.05$), PERMANOVA pairwise comparisons were carried out. Distance-based redundancy linear modeling (DISTLM) with a test of marginality in PRIMER was also performed to account for the contribution of environmental parameters and gonad cycle in explaining the total observed variance in the transcriptional profiles. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. BEST/BioEnV routine in PRIMER 6 was also carried out using a Spearman rank correlation to identify the best environmental variables that explained the observed patterns of gene transcriptions (999 permutations).

For microbiome analyses, raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME 2 (Bolyen et al., 2019); https://qiime2.org). High-quality reads were clustered into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using the SILVA database as a reference (Quast et al., 2013). Unassigned sequences and those assigned to eukaryotes (i.e. chloroplasts and mitochondrial ones) were discarded. Beta diversity was estimated by computing unweighted UniFrac distance. All statistical analyses was performed using R software version (R Development Core Team, 2018). ASVs were filtered for prevalence, retaining only ASV showing a relative abundance >1% in at least 10% of samples. UniFrac distances were plotted using the vegan package, and permutation test pseudo-F ratios (function adonis in the

vagan package) was computed to test the significance of data separation in the principal coordinate's analysis (PCoA). False discovery rate (FDR) (function p.adjust in the stats package) was used to adjust p-values, and a P-value ≤ 0.05 was considered as statistically significant.

3. RESULTS

3.1. Mussel transcriptional profiles in digestive glands

Variations of gene transcriptional profiles between sexes or across season are reported in Fig 2. Results from PERMANOVA analyses demonstrated that the single factors season and sex had a significant effect on the whole dataset (P(perm) < 0.05; Table 1). Furthermore, PERMANOVA analysis showed a significant interaction (P(perm) < 0.05; Table 1) between the factors. The BEST/BioEnV analysis on showed the environmental variables that best correlated with the overall transcriptional dataset (Table S2).

Table 1. PERMANOVA results on transcriptional profiles (998 permutations).

Source	df	Pseudo-F	P(perm)
Season	2	21.897	**0.001**
Sex	1	32.611	**0.001**
Season x Sex	2	7.2798	**0.001**

df: degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P(perm): probability of pseudo-F.

Significantly different expression levels between males and females are observed for *mt20*, *abcb* and *hex* (P < 0.05). All gene products showed complex transcriptional patterns across season in both males and females (Fig 2), with a tendency to increased (*amil*, *lys*, *mt20*, *abcb*, *cat*, *sod*, *hex*, *ctsl*, *gusb*,) or decreased (*pk*, *idp*,) expression from winter to

summer. DISTLM analyses performed on separate female and male datasets by considering environmental parameters and gonadal maturation level (assessed through *VCL/VERL* expression profiling) showed that in females environmental parameters and gonadal maturation mostly explained the observed transcriptional profiles ($P < 0.05$; Fig 3). In males, only environmental variables give a significant contribution ($P < 0.05$; Fig 3). Among these explaining variables, the BEST/BioEnV analysis showed that temperature and gonad maturation significantly correlated with transcriptional profiles of females, while salinity, surface oxygen and transparency significantly correlated with transcriptional profiles of males (Table S2).

3.2. Microbiome analysis

The compositional microbiota structure of 41 mussels along the annual cycle was obtained by NGS sequencing of the V3–V4 hypervariable region of the of the 16S rRNA gene. A total number of 623000 high quality reads were obtained (mean per sample ± SD, 15 195 ± 11 581) and clustered in 614 ASVs at 97% identity. To explore overall differences in the microbiota composition between samples, an unweighted Unifrac-based PCoA of the correspondent compositional profiles was carried out.

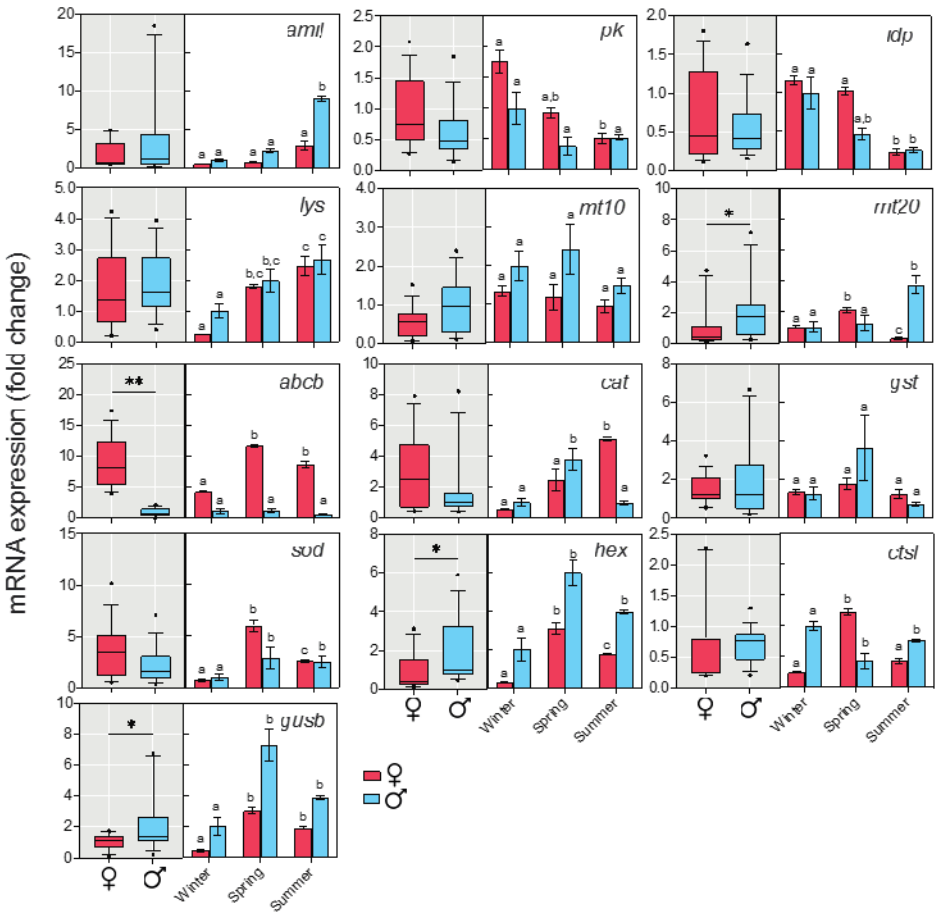


Fig 2. Transcriptional profiles of metabolic (*amil, pk, idp*), cytoprotective/detoxification), (*lys, mt10, mt20, abcb*), antioxidant (*cat, gst, sod*), and lysosomal (*hex, ctsl, gusb*) mRNAs in females and males farmed Mediterranean mussels from the North Adriatic Sea. For each target transcript box plots (grey area) report overall expression levels in females *vs* males (median, upper and lower quartiles; N =18), while bar plots (white area) show transcriptional profiles across the sampling seasons and for the different genders (mean ± sem; N = 6). In box plots: *P<0.05 male *vs* female. In bar plots: different letters indicate statistical differences between samples within male or female sample groups (P < 0.05).

According to our findings, mussel samples clustered in 3 groups which correspond to the collection season (permutation test with pseudo-F ratios, P-value ≤.02) (Fig. 5A). From the compositional point of view, Firmicutes characterized winter samples, while Tenericutes

were most abundant in the summer. Conversely, Proteobacteria appeared to be constant along the year (Fig. 5B). Interestingly, besides seasonal variation, the samples also tend to segregate (Fig. 5A) according to mussel sex (permutation test with pseudo-F ratios, P-value

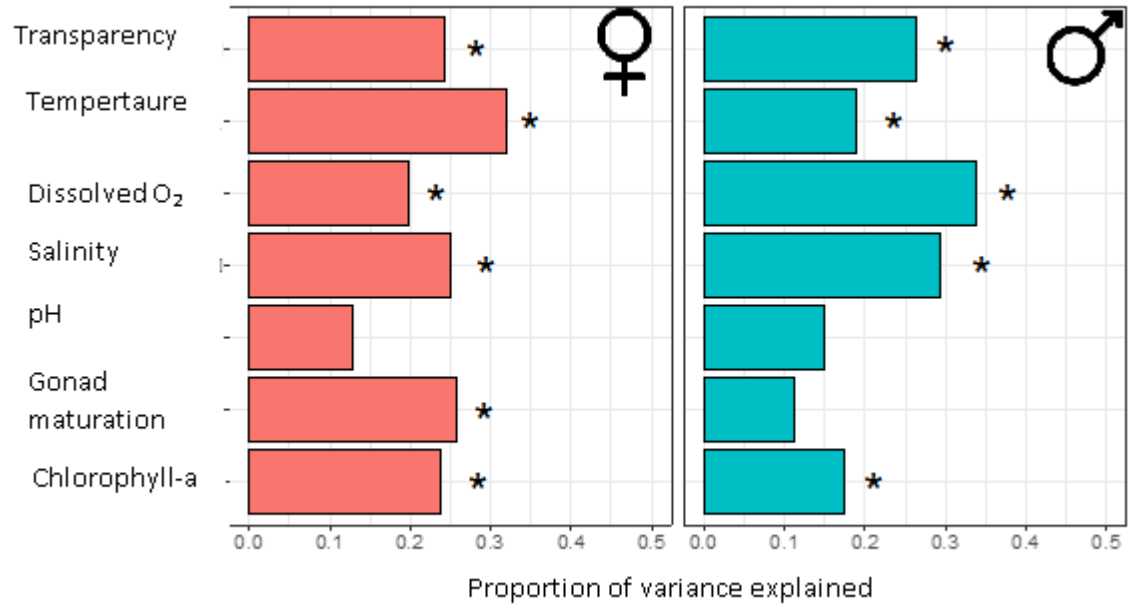


Fig 3. DISTLM analysis to explore trends of biological parameters with environmental variables in females and males sample groups. Results from the test of marginality related to the distance-based redundancy (DISTLM) analysis showing contribution of each environmental variable to the total variance observed in female and male datasets of gene transcription profiles. Gonad maturation profiles reported in Fig 1 have been included as a predictor variable. DISTLM used the BEST selection procedure and adjusted R^2 selection criteria. Asterisks indicate level of statistical significance related to the result (*P < 0.05).

≤.2), suggesting sex-specific differences in the composition of *M. galloprovincialis* microbial ecosystems. Particularly, as shown in Fig. 5B, males are most abundant in Cyanobacteria (6% ± 11.4% in male, 2.1% ± 7.6% in female), Planctomycetes (5.3% ± 7.7% in male 0.6% ± 1.5% in female) and Chlamydiae (2% ± 4.7% in male, 0.6% ± 1.5% in female), while females show an increase in Firmicutes (16.1% ± 22.5% in male, 19.7% ± 26.9% in female), Bacteroidetes (2.7% ± 3.4% in male, 8.9% ± 16.2% in female) and Actinobacteria (4.7% ± 6.6% in male, 5.9% ± 12.7% in female).

4. DISCUSSION

Data reported in this study show the influence of both seasonality and gender bias on transcriptional profiles and microbiota composition of *M. galloprovincialis* from the Northwestern Adriatic Sea.

Season related fluctuations of molecular and biochemical biomarkers in mussels can be expected, as reported by a relevant amount of scientific evidence on this topic (Balbi et al., 2017c; Benito et al., 2019; Leiniö and Lehtonen, 2005), likely depending on seawater temperature and salinity variations, which are amongst the main drivers of physiological regulation for mussels and other intertidal marine invertebrates (Lockwood et al., 2015). Indeed, according to the BEST/BioEnv analysis performed on the whole transcriptional dataset, temperature and salinity are the best correlated environmental variables with the observed biological outcomes, together with pH and chlorophyll-a variations. This finding suggests a more complex interaction with the environmental conditions provided by the sampling area in the Northwestern Adriatic Sea, which are characterized by a large river runoff from the Italian border and by highly variable meteorological conditions (Alvisi and Cozzi, 2016). DG microbiome composition also followed a seasonal pattern, with Firmicutes and Tenericutes characterizing winter and summer samples, respectively.

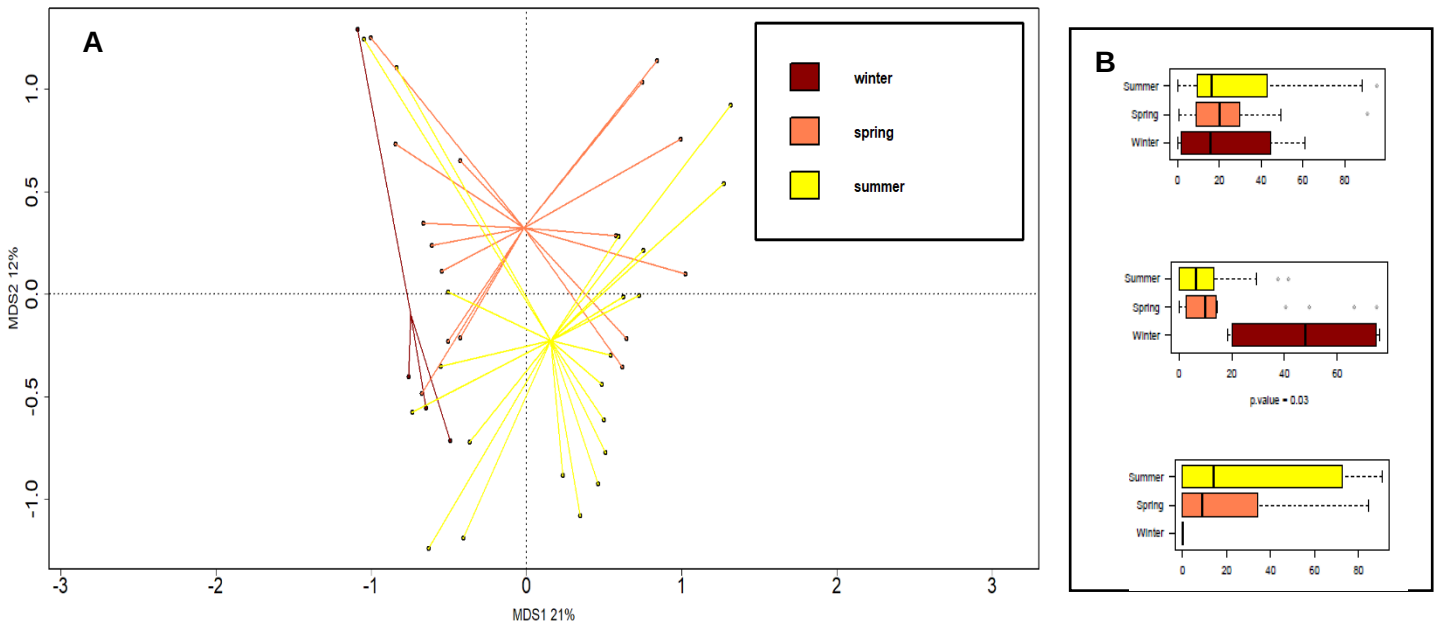


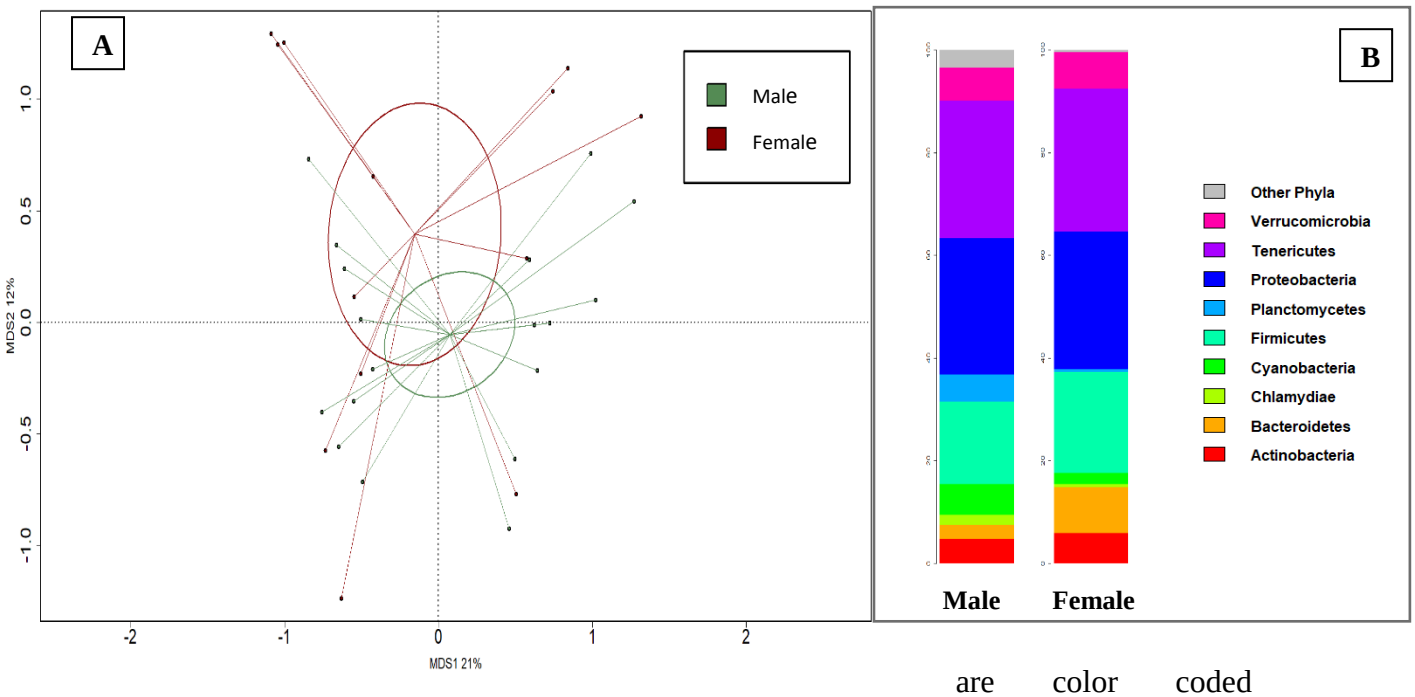
Figure 4. Variation of the *M. galloprovincialis* microbiota according to seasonality. (A) PCoA based on unweighted UniFrac distances between samples compositional profiles, samples are color coded according to seasonality. (B). Boxplot showing dominants phyla in the different seasons.

The overall seasonal pattern of gene transcription shows a general increasing expression from winter to summer, along with temperature rising, except for transcripts encoding metabolic enzymes, that show both increasing (*amyl*) and decreasing (*pk*, *idp*) expressions across season. Amylase is a key enzyme in carbohydrate metabolism; pyruvate kinases and isocitrate dehydrogenases are engaged in channeling glycolitic substrates towards aerobic metabolic pathways (Canesi et al., 1999; Liu et al., 2017). On the whole, the relative expression patterns of these gene products suggest a lower aerobic capacity of the mussels in summer, or, alternatively, an enhanced occurrence of substrates for anaerobic metabolism. Interestingly, this host response to seasonal changes parallel the observed variations of DG microbiome composition. At low (winter) temperatures, the mussel DG microbiome enriches fiber fermenting anaerobes belonging to Firmicutes, which generally populate digestive tract of terrestrial and marine animals (Musella et al., 2020; Rausch et al., 2019), complementing the oxidative propensity of the host overall metabolic layout. With raised temperatures (summer), the DG microbiome becomes characterized by Tenericutes, a taxon that includes non-peptogenic parasites living in close association (and dependence) with host cells (Lee et al., 2018), thus not competing directly with substrates for supporting an anaerobic host metabolism.

Results of this study further demonstrate sex related expression of some gene transcripts and of microbiota composition. Generally speaking, females and males differ for their expression profiles across season. Both DISTLM and BEST/BioEnV analyses indicate that in males transcriptional profiles seem related only to environmental variables, mainly to salinity, surface oxygen, and transparency, whereas in females seawater surface temperature

and gonad maturation are the best correlated factors and explained most of the variance of the transcriptional dataset. Therefore, results of this study agree with previous findings assessing that season-related differences in the biomarker responses of mussels between females and males may reflect the progression of the reproductive cycle (Blanco-Rayón et al., 2020).

Figure 5. Variation of the *M. galloprovincialis* microbiota composition according to sex. **A.** PCoA based on unweighted UniFrac distances between samples compositional profiles, samples



are color coded according to sex. **B**. Barplot showing phylum-level mean r.a % in male and female samples.

Some gene products displayed significantly different overall expression levels between sexes. The most remarkable difference is observed for the *ABCB* transcript encoding the mussel P-glycoprotein (P-gp), whose expression is significantly higher in females than in males. ABC transporters are ATP-dependent active transporters pumping out from cells both endogenous chemicals and xenobiotics, thus preventing their accumulation and toxic effects (Bard, 2000). Besides the fact that these proteins are considered a first-tier defense against chemical toxicities, their role in mammalian oocyte maturation has been postulated (Bloise et

al., 2016). It is worth noting that well detectable levels of *ABCB* mRNA were detected in both unfertilized and fertilized mussel oocytes after spawning (Franzellitti et al., 2017), suggesting a similar function in mussels. The maternal origin in mussel oocytes of P-gp/ABCB and of other gene products involved in the first stages of embryo development and protection (Balbi et al., 2016) likely requires that females do invest a larger proportion of the available energy in gametogenesis than males. It has been suggested that in females such sex-specific processes may impair the induction of cytoprotective mechanisms, altering their capacity to cope with environmental stressors (Bedulina et al., 2020). This observed differential expression and season regulation of a cytoprotective mechanism corroborates previous investigations showing sex related differences in pollutant bioaccumulation and in biological responses to pollutants (Blanco-Rayón et al., 2020).

5. CONCLUSIONS

Results of this study integrate previous investigations on season- and sex- related differences in mussel responsiveness to environmental stressors by showing the differential regulation of gene transcripts that may underpin such physiological responses under the influence of natural environmental variability and gonadal development, and further exploring the connection between the health and productivity of farmed mussels and the environmental quality. Moreover, the application of this approach to farmed mussel broodstock may provide a means to establish advanced early-warning biomarkers of effect tailored to commercially relevant physiological traits (i.e. growth, reproduction, resistance to bacteria) which can be employed to monitor the physiological performances of the organisms in different farming areas and across different seasons.

Supplementary data to this article can be found in the Supplement 5

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Reference genes selection for transcriptional analyses in *Mytilus galloprovincialis* under natural field conditions

Manuscript under preparation

Abstract

It is a great challenge to understand the physiological acclimation of mussels in the field due to the presence of multiple stressors, inter-individual variability, and further confounding factors (biotic, abiotic). However, intensive investigation of the abiotic and biotic dependent expression patterns of its genes, accurate quantification of the target gene transcription level is a fundamental step for marine mussels residing in nature. Normalization of the gene expression using an appropriate reference gene (RG) is important to have a reliable mRNA level analyzed by real-time PCR (qPCR). In this study, we assessed the expression stability of six commonly employed RGs, namely β-actin (ACT), tubulin (TUB), α helicase (HEL), elongation factor 1 α (EF1), 18s ribosomal RNA (18s), and 28s ribosomal RNA (28s) under abiotic (season, location) and biotic (gender bias) conditions in the field. The expression stability was calculated using multiple statistical approaches, and it showed relatively consistent results, with 18s, 28s, and TUB being the most stable RGs across all studied conditions, but in different ranking order. However, spring season exhibited different stability status in comparison to winter and summer, suggesting that their expression might be affected by seasonal factors. Combining 28S rRNA and 18S rRNA appears the best strategy for accurate qPCR data normalization on the overall expression trends of target gene in the digestive gland of the mussels under natural conditions in the field.

Keywords: gender, season, location, 18s, 28s, TU, reference gene

1. INTRODUCTION

Gene expression is widely used to understand organism responses to environmental pressures and to have a great understanding of physiological responses since it has identified as an early warning signal of the organism before establishing phenotypic responses (Counihan et al., 2019; Diaz de Cerio et al., 2017; Izagirre et al., 2014). Quantitative real-time polymerase chain reaction (qPCR) is the common technique for the measurement of messenger RNA (mRNA) levels of genes expressed across different experimental conditions. However, this technique is generally subjected to errors like RNA extraction and quality, pipetting errors, reverse transcription efficiency, and the inhibitors' presence (Andersen et al., 2004; Vandesompele et al., 2002). Normalization with internal control or housekeeping gene/reference gene (RG) to adjust these technical errors in the qPCR workflow is recommended (Boonphakdee et al., 2019; Volland et al., 2017). An ideal housekeeping gene should be expressed continuously regardless of any conditions like tissue type, developmental stages, and experimental setup (Wong and Medrano, 2005). So far, a universal reference gene that is constant under all experimental conditions is not established; thus, it is needed to carefully choice of one or more reference genes according to the tissue type or the given experimental setup (Soares-souza, 2017).

According to the review of Volland et al. (2017), more than 60 % of studies on bivalves have no validated RGs; however, only 25% documented its validation protocol out of the remaining 40 % of studies. It is evidenced that RG stability patterns in *Mytilus* sp. differ between their tissues exposed to algal toxins (Lacroix et al., 2014; Martínez-Escauriaza et al., 2018), reproductive stages before and after exposure to endocrine disrupters (Cubero-Leon et al., 2012), pathology conditions (Rebeca Moreira 2014). The same species had shown different RG stability patterns in their tissues and life stages (Feng et al., 2013).

It is hard for scientists to study physiological changes of mussel under natural conditions for a single stressor or environmental parameter because the physiological outcomes are influenced by multiple interacting variables, including anthropic and environmental stressors (Lacroix et al., 2014). In the field, gene expressions of mussels are challenged by factors like temperature, food availability, and intrinsic characteristic like life stage, reproduction stage, or gender (Banni et al., 2011; Benito et al., 2019; Jarque et al., 2014; Okumuş and Stirling, 1998; Sheehan and Power, 1999). Although mussels had been surpassing bad environmental conditions, there is a lack of information on their physiological adaptation across the environmental pressures under the natural environment.

qPCR data normalization using appropriate reference gene (RG) is important to have reliable mRNA levels. However, no systematic investigation on the RG stability used in *Mytilus galloprovincialis (*Mediterranean mussel) residing in the natural environment has been performed thus far. It is vital to have stable RGs for a great understanding of the natural variability of the transcriptional profile along with the seasonal, geographical variations, and gender-based to avoid data misinterpretation (Lacroix et al., 2014).

This study was performed to explore the variability of commonly employed eference genes for quantifying target gene expression by qPCR in *M. galloprovincialis* under field conditions. To this aim, extensive reference gene stability analysis using multiple statistical approaches was performed on mussels collected over different seasons, geographical locations, and considering reproduction stage and gender. The digestive gland was selected as the target tissue, because it plays a major role in food digestion, detoxification (Izagirre and Marigómez, 2009) and well known as an immune organ, and the same time it is widely employed in environmental studies due to presence of a battery of biomarkers to stress (Laura Canesi et al., 2010; Liu et al., 2014), and, recognized as the best organ to perceive physiological changes in the natural environment (Canesi et al., 2010; Ivanković et al., 2005).

2. METHOD

2.1 Collection of mussels

This study was carried out as part of our study on basal expression of transcript involved in physiological processes adult mussels resides in the natural environmental gradients of physical/chemical parameters. *Mytilus galloprovincialis* (Mediterranean mussels) has been selected because it is one of the most intensively cultured bivalves in the Western Adriatic Sea region, and contributing a significant part to the area's economic sector (Minarelli et al., 2018). The mussel farms are scattered from north to southern part of the region, and the peculiar characteristics of the Adriatic Sea make favorable natural conditions for the production and maturation of mussels (Tamburini et al., 2020). From north to south part of the Adriatic Sea display geographical, chemical and environmental gradients (Kraus et al., 2019), thus create ideal field laboratories to infer effects of different natural treatments factors. The experimental setup was implemented to have three differential factors: location, season, and gender.

Gender effects. Five sampling campaigns were performed from the commercial mussel farm located in the Northern Adriatic Sea by professional fishermen of the "Cooperativa Pro.mo.ittica" (Cesenatico, Italy, figure 1) to examine the stability of the selected reference genes across the gender. Mussel samples were collected once a month, from March to July 2018. Fifteen mussels of commercial size (5–7 cm in length) were immediately stored in coolers (+4 °C) and transferred to the laboratory. In the laboratory, the mussels were cleaned and gently washed and then dissected under sterile conditions to collect the digestive gland and mantle. Sex determination was done through the PCR-based identification (Fraser et al., 2016) by employing of the female-specific vitelline envelope receptor for lysine (VERL) and male-specific, vitelline coat lysine (VCL) transcripts in

mantle/gonad complex (Hines et al., 2007; Sedik et al., 2010). qPCR based protocol was performed for each mantle/gonad complex using primer pairs (Table S1) to obtain the threshold cycle (CT). Threshold cycle (CT) values were determined by setting a constant baseline. Sex was determined by calculating the intra animal ΔCT as CT (VCL) – CT (VERL) (Anantharaman and Craft, 2012); negative values indicate males and positive values indicate females.

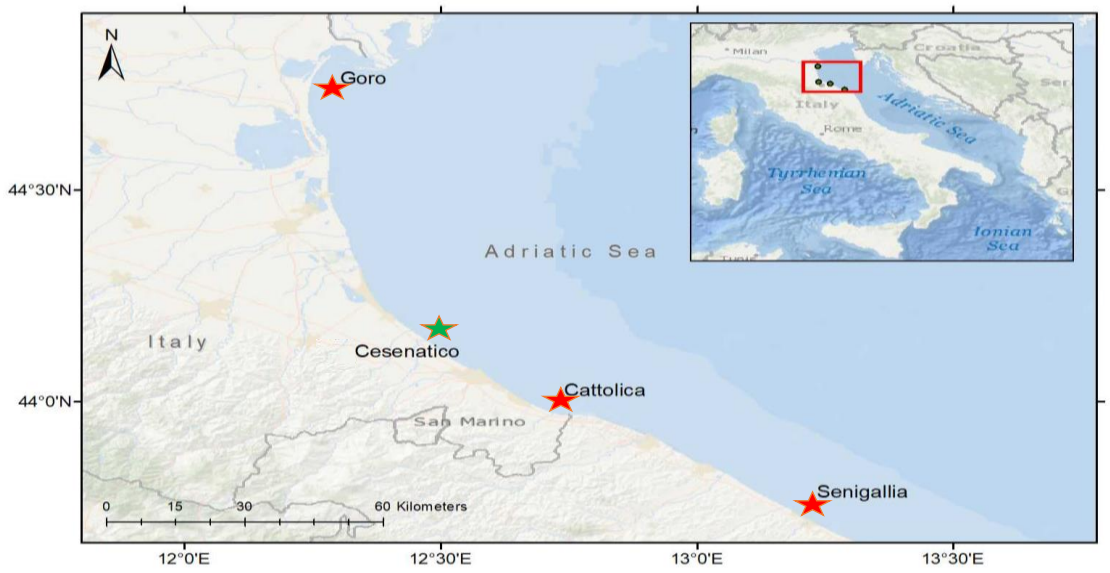

Fig 1. Study area, location of the mussel farms assessed in this study in the NorthWestern Adriatic Sea (Italy).

Location and season effects. Mussels were collected from the three locations, namely: Goro, Cattolica, and Senigallia (figure 1). The farms at Goro, Cattolica, and Senigallia represent from the north to the south coastal part of the western Adriatic Sea. Those farms are generally well-established in the area. Other than the location factor, to stimulate the seasonal effects, sampling was carried out in consecutive three seasons, including winter, spring, and summer in each location. Each sample consisted of twelve mussels of commercial size (5–7 cm in length) and immediately transferred to the laboratory for further analysis.

2.2 Selection of reference gene

Commonly available six housekeeping genes β-actin (ACT), tubulin (TUB), *α* helicase (HEL), elongation factor 1 *alpha* (EF1), 18s ribosomal RNA(18s), and 28s ribosomal RNA (28s) were selected to identify the suitable reference gene for qPCR in *M. galloprovincialis* (Cubero-Leon et al., 2012; Lacroix et al., 2014; Martínez-Escauriaza et al., 2018; Moreira et al., 2015; Young et al., 2019). The details of the six selected reference genes were shown in Table1.

Table 1. **Candidate reference genes and, primers, amplicon lengths, amplification efficiencies, GeneBank accession numbers, functions, and references.**

Gene	Primers	Amplicon size(bp)	Amplification Efficiency (%)	Function	Accession number	References
18S	5'- TCGATGGTACGTGATATGCC -3' 5'- CGTTTCTCATGCTCCCTCTC -3'	90	95	Protein synthesis	L33451	(Dondero et al., 2005)
28S	5'- AGCCACTGCTTGCAGTTCTC -3' 5'- ACTCGCGCACATGTTAGACTC -3'	142	94	Protein synthesis	DQ158078	(Ciocan et al., 2011)
ACT	5'- GTGTGATGTCATATCCGTAAGGA -3' 5'- GCTTGGAGCAAGTGCTGTGA -3'	120	114	Cell motility, structure, and integrity	AF157491	(Banni et al., 2011)
TUB	5'- TTGCAACCATCAAGACCAAG -3' 5'- TGCAGACGGCTCTCTGT -3'	135	102	Microtubule element – cell structure	HM537081	(Cubero-Leon et al., 2012)
EF1	5'- CGTTTTGCTGTCCGAGACATG -3' 5'- CCACGCCTCACATCATTTCTTG -3'	135	99	protein synthesis	AB162021	(Ciocan et al., 2011)
HEL	5'- GCACTCATCAGAAGAAGGTGGC -3' 5'- GCTCTCACTTGTGAAGGGTGAC -3'	129	132	RNA processing	DQ158075	(Cubero-Leon et al., 2012)
	Sex specific genes					
VCL	AGAGCTGTTTTGGCCACAGT TTGCGTTTGACATGGTTGAT		100	vitelline coat lysin	FM995162	(Anantharaman and Craft, 2012)
VERL	CCGAAGGAAATGGAACTGAAA CCCTGCAATCGTATGGAATC		100	vitelline envelope receptor for lysin	FM995161	(Anantharaman and Craft, 2012)

2.3 *RNA extraction and cDNA preparation from mussel tissues*

For each animal, 100mg of digestive gland tissue were homogenized in a 1ml of the TRI Reagent (Sigma Aldrich, Milan, Italy), and total RNA was extracted using the DirectZol kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. RNA purity and concentration were confirmed using the Qubit system with the Qubit RNA assay kit (Thermo Scientific, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. The analysis of UV absorbance spectra of the samples (λ = 200 – 340 nm) allowed the calculation of Absorbance (A) ratio A260/A280 addressing the occurrence of protein contaminations (cut-off values > 1.8 and < 2.0), and the ratio A260/A230 addressing the occurrence of contaminants that may be present in the samples, such as guanidine thiocyanate, which is a component of the TRI Reagent (cut-off value > 1.7). The first-strand cDNA for each sample was synthesized from 1 µg total RNA using the iScript supermix (Bio-Rad Laboratories, Milan, Italy) following the manufacturer's protocol.

2.4. *Real-time PCR (qPCR) analysis*

Expressions of interested RGs were assessed by real-time PCR (qPCR) using primer pairs reported in Table 1. Briefly, assays were performed in duplicate using 2 µL of diluted cDNA, and 0.2 µM of specific primers, and 5 µL of SYBR Green master mixes with ROX (Bio- Rad Laboratories, Milan, Italy) in a total volume of 10 µL. Each PCR run included the control lacking cDNA sample and water controls to determine target cDNA amplification's specificity. Amplifications were performed in a StepOne real-time PCR system apparatus (Thermo Fisher, Milan, Italy) using a standard “fast mode” thermal protocol. For each target mRNA, melting curves were utilized to verify specificity of the amplified products and the absence of artifacts. The amplification efficiency of each primer pair was calculated using a dilution series of cDNA (Table 1). Technical replicates have been performed both within

each run/plate (samples in duplicate) and between different runs/plates (samples replicated on different plates). Equal loadings within each qPCR reaction were ensured by checking the amounts of each cDNA sample using the Qubit system with Qubit dsDNA HS assay kit (Thermo Scientific, Milan, Italy).The protocols employed for qPCR assays are reported in previous studies (Balbi et al., 2016).

2.5 *Analysis of reference gene expression*

The stability of the selected six reference genes was estimated using Microsoft Excel-based software applications; NormFinder (Andersen et al., 2004), geNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004), and a comparative Ct method using the DataAssist software (Life Technologies) (Silver et al., 2006). These software packages are freely available to download and have been widely used to identify suitable reference genes (Boonphakdee et al., 2019; De Spiegelaere et al., 2015; Volland et al., 2017). To have an overall final ranking from the above four methods, a freely available RefFinder web-based tool was used (https://www.heartcure.com.au/reffinder/).

2.6 *Statistics*

CT values were compared between the different sampling groups using non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney U-test ($p < 0.05$), after deviations from parametric ANOVA assumptions being verified (Normality: Shapiro-Wilk's test; equal variance: Bartlett's test). These statistical analyses were performed using GraphPad Prism 6 software (GraphPad Inc.).

3. RESULTS

3.1 *Expression patterns of reference genes*

The mRNA expression of the six candidate reference genes (Table 1) was assessed in *Mytilus galloprovincialis* considering gender, location, and season. For gender, mussel samples were collected from a farm off Cesenatico in 2018, and samples from Goro, Cattolica, and Senigallia were considered to assess location and seasonal -based analysis.

Descriptive statistics for each candidate reference gene is reported in Table 2. 18s showed the lowest average CT values (mean CT = 13.08), while HEL showed the highest lowest expression (mean CT = 28.96). Among the genes that were tested for the three different conditions, the ribosomal RNA genes (18S and 28S) showed the lowest standard deviation (SD) (SD = ± 1.298 and ± 1.945, respectively).

Table 2. Selected candidate reference gene and their parameters derived from qPCR analyses. Gene abbreviations are as reported in Table 1. N: total number of samples; mean CT: mean CT values; SD: standard deviations of the CT values; CV(%): coefficients of variation of the CT values.

Gene	N	Mean C_T	± SD	CV (%)
ACT	128	26.06	2.354	9.03%
TUB	128	20.96	2.019	9.64%
HEL	128	28.96	2.51	8.67%
EF1	128	19.41	2.857	14.72%
18s	128	13.08	1.298	9.93%
28s	128	20.79	1.945	9.36%

According to the overall CT values, there is variability among all the examined candidate reference genes (Table 2). Here the most stable and variable transcripts being 18s

and EF1, respectively. When considering interfactors variability, all transcripts showed the most stable (P>0.05) expression levels across the different factors (Fig. 2). The results obtained after statistical analyses are not shown here (**See S1, S6 supplement File**).

Considering the single factor, the tested gene transcription levels showed the highest stability (P>0.05) across gender (Fig. 3A; Table 3). Variations in CT values, which ranged between 13.16 ± 0.311 (mean ± SE of 18s values in the male) (Fig. 3A) and 29.11 ± 0.408 (mean ± SE of HEL in the male) (Fig. 3A), were observed among the different genes and sex. Furthermore, each studied gene exhibited statistically (P>0.05) similar expression across the three locations (Fig. 3B; Table 3). Similar to gender, relatively higher mRNA expression was observed in 18s for three locations while HEL is the lowest gene (Fig. 3B)

However, each reference genes showed significant transcript levels (P<0.05) between the three seasons (winter, spring, and summer, Fig. 3C). The lowest and highest CT values are represented by 18s and HEL, respectively (Fig. 3C), which was mainly influenced by the difference in seasonal status, as shown in Fig. 3C).

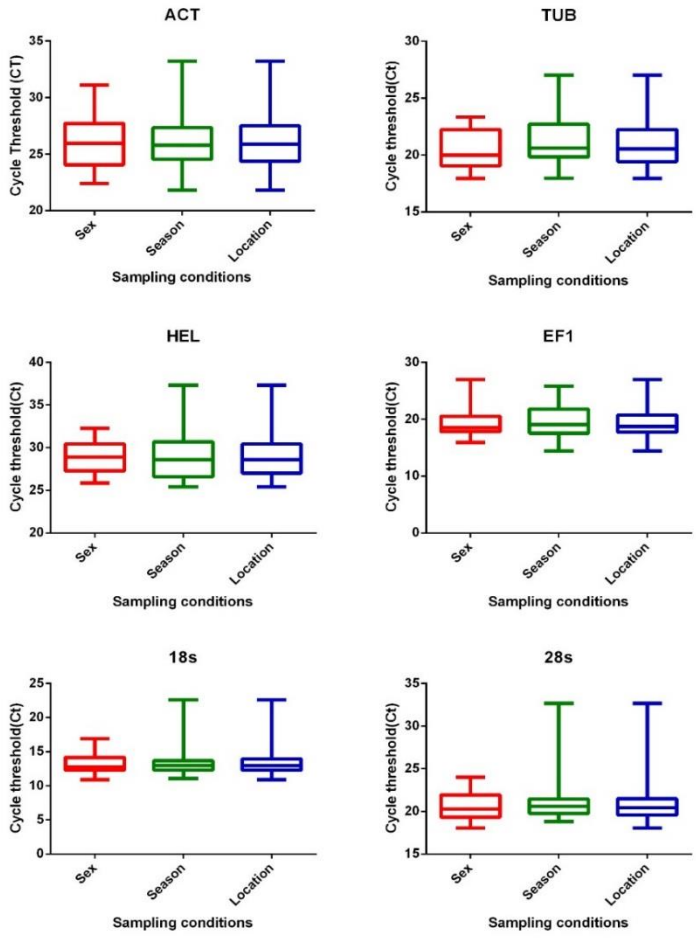

Fig 2. Intergroup transcript level variability of each candidate reference gene product in digestive gland from Mediterranean mussel. Data are based on threshold cycle (CT) values of candidate reference transcripts on digestive gland samples from mussels at different conditions. Box-and-whisker plots represent median, upper and lower quartiles (n (sex) = 28, n (season)=64, n (location)-64).

3.2 *Analyses of candidate reference transcript stability*

To identify the most robust reference gene within the digestive glands of Mediterranean mussels for qPCR analysis considering the three different factors (gender, location, and season), four algorithms were used (BestKeeper, NormFinder and ΔCT method) and RefFinder tool to individually analyze the expression stability of those genes.

The software calculated gene expression stability and then ranked them based on their stability values (M). The candidate RGs with a small stability value identified that were more stable genes. The data from the analysis using the four program and RefFinder tool programs are summarized in Table 3.

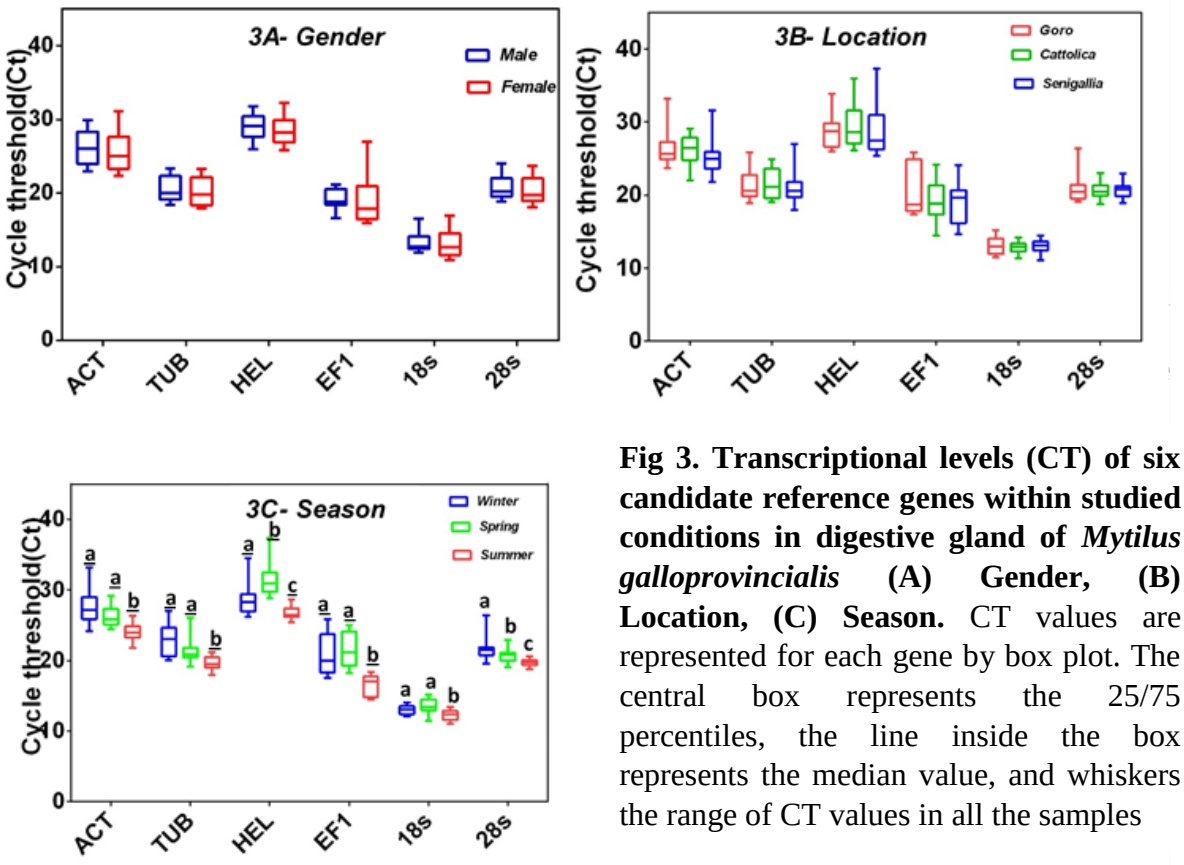

Fig 3. Transcriptional levels (CT) of six candidate reference genes within studied conditions in digestive gland of *Mytilus galloprovincialis* (A) Gender, (B) Location, (C) Season. CT values are represented for each gene by box plot. The central box represents the 25/75 percentiles, the line inside the box represents the median value, and whiskers the range of CT values in all the samples

Table 3**. Stability rankings of the candidate reference gene products obtained with different computational methods for different**. Stability values obtained by each method are shown in parenthesis. The gene products are ranked from the most stable (1) to the least stable (6).

Sex	**Comprehensive ranking (RefFinder)**	**Comparative delta-CT**	**BestKeeper**	**NormFinder**	**geNorm**
1	28s(1.68)	TUB(1.5)	18s(1.23)	TUB(0.623)	18s|28s(0.901)
2	TUB(1.73)	28s(1.63)	28s(1.30)	28s(1.014)	
3	18s(1.73)	18s(1.64)	TUB(1.51)	18s(1.056)	TUB(1.291)
4	ACT(4.43)	ACT(1.78)	HEL(1.53)	ACT(1.285)	ACT(1.411)
5	HEL(4.73)	HEL(1.94)	EF1(1.77)	HEL(1.532)	HEL(1.576)
6	EF1(5.73)	EF1(2.18)	ACT(1.96)	EF1(1.867)	EF1(1.778)
Season					
1	18s(1.57)	TUB(1.88)	18s(1.42)	TUB(1.044)	18s|28s(0.899)
2	TUB(1.73)	18s(1.96)	28s(1.59)	ACT(1.259)	
3	28s(2.38)	ACT(2.00)	TUB(1.68)	18s(1.336)	TUB(1.444)
4	ACT(3.13)	28s(2.01)	ACT(1.79)	28s(1.460)	ACT(1.627)
5	HEL(5.00)	HEL(2.18)	HEL(2.29)	HEL(1.541)	HEL(1.845)
6	EF1(6.00)	EF1(2.65)	EF1(2.61)	EF1(2.310)	EF1(2.113)
location					
1	18s(1.57)	TUB(1.88)	18s(1.33)	TUB(0.916)	18s|28s(0.914)
2	TUB(1.73)	18s(1.96)	28s(1.46)	ACT(1.212)	
3	28s(2.38)	ACT(2.00)	TUB(1.59)	18s(1.282)	TUB(1.385)
4	ACT(3.13)	28s(2.01)	ACT(1.87)	28s(1.295)	ACT(1.556)
5	HEL(5.00)	HEL(2.18)	HEL(1.95)	HEL(1.527)	HEL(1.745)
6	EF1(6.00)	EF1(2.65)	EF1(2.25)	EF1(2.111)	EF1(1.981)
whole dataset					
1	18s(1.41)	TUB(1.74)	18s(1.36)	TUB(0.965)	18s|28s(0.907)
2	TUB(1.73)	18s(1.83)	28s(1.50)	18s(1.251)	
3	28s(2.06)	28s(1.86)	TUB(1.62)	ACT(1.282)	TUB(1.403)
4	ACT(4.00)	ACT(1.92)	ACT(1.84)	28s(1.343)	ACT(1.579)
5	HEL(5.00)	HEL(2.08)	HEL(2.07)	HEL(1.525)	HEL(1.777)
6	EF1(6.00)	EF1(2.45)	EF1(2.38)	EF1(2.173)	EF1(2.023)

Stability values (M) calculated by geNorm for three of the candidate genes were higher than the cut-off value of 1.50, and only those of three genes 18s, 28s, and TUB were a little bit lower than 1.50. Based on the RefFinder, the same stability ranking order of reference gene across each factor was: 18s, TUB, 28s, ACT, HEL, and EF1 except gender analysis. Considering all four algorithms and RefFinder tools, 18s, TUB, and 28s are the most stable reference genes for each factor and whole data set with different ranking order.

Other than the pooled conditions, data within individual factors such as each season, each gender, and each location were analyzed using the same logarithms. Those results are not shown here (**See S6 supplement File**).

For both male and female samples, the rankings of reference gene stability were similarly based on the ΔCT method and NormFinder, showing TUB is the topmost stable gene (**See S2 Table, S6 supplement File**). According to the BestKeeper, 18s and 28s were the best stable gene for males and females, respectively. Results obtained from geNorm methods confirmed both 18s and 28s were the most stable genes for male and female samples. Integrating the results of all four methods, RefFinder ranked 18s as highest while EF1 was the lowest for male and female samples.

For Cattolica and Senigallia, the top most stable reference gene was TUB according to the ΔCT method and NormFinder (**See S3 Table, S6 supplement File**). However, for Goro, ΔCT method and NormFinder had shown the ACT and 28s are the most stable two genes. Analysis using the geNorm method exhibited that 18s and 28s were the top two reference genes for each location. However, BestKeeper showed that 28s, 18s, and ACT, were the most stable reference genes in Goro, Cattolica, and Senigallia, respectively. According to the RefFinder, the ranking of six selected reference genes from the most stable to the least stable was ranked as Goro (28s, ACT, TUB,18s, HEL, EF1), Cattolica (TUB, 18s, 28s, ACT, EF1, HEL), Senigallia (TUB, 18s,28s, HEL, ACT, EF1).

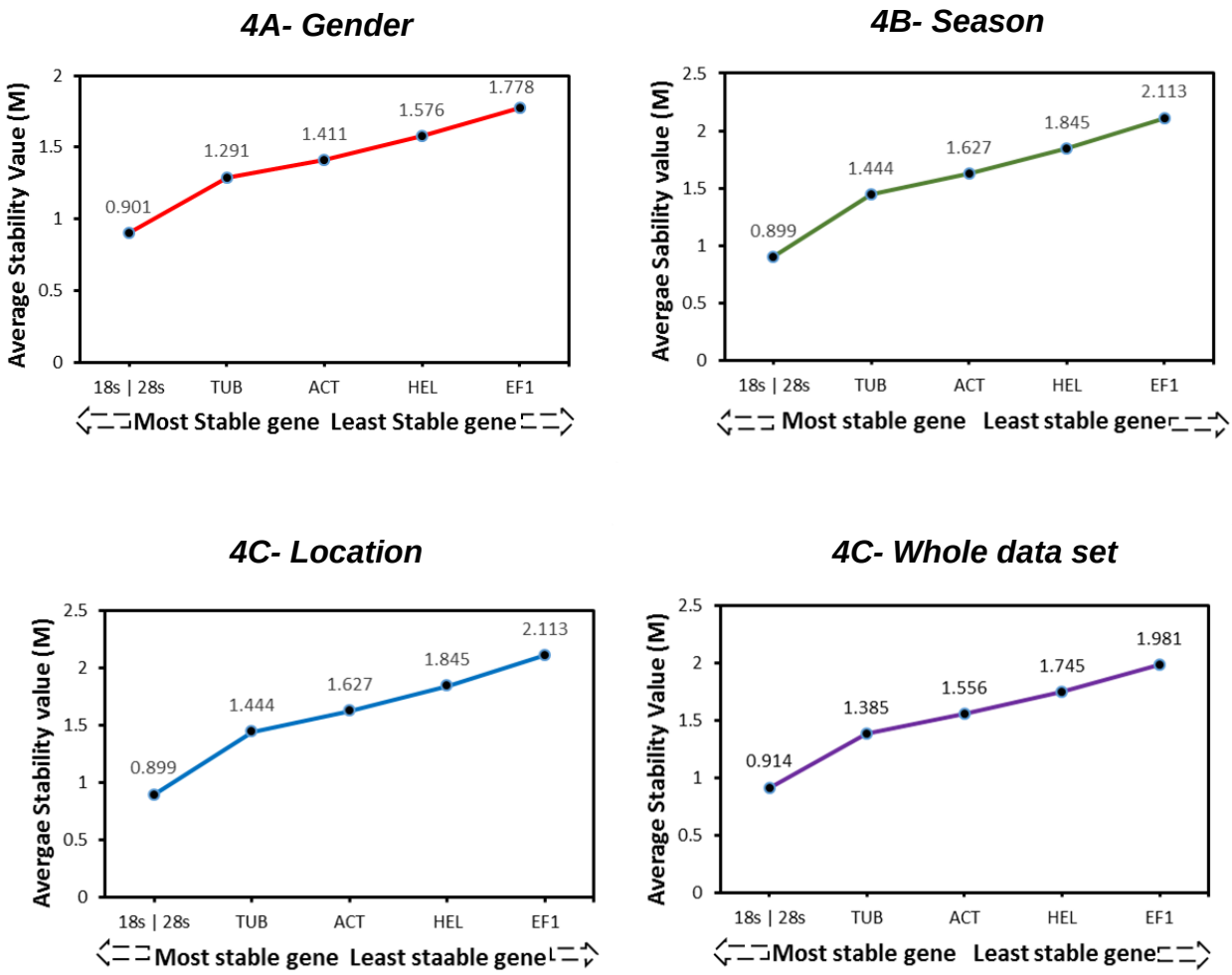

Fig. 4. Selection of the most suitable reference genes for normalization in difference conditions (4A-Gender, 4B-Location, 4C-Season, and 4D- whole data set as calculated using geNorm . The x axis from left to right indicates the ranking of the genes according to their expression stability (M). The lower stability value indicates more stable reference genes across the different conditions: gender, season, location and whole data set.

Interestingly, it has shown that reference gene stability was different across each season (**See S4 Table, S6 supplement File**). According to the ΔCT method and NormFinder results, HEL and TUB were the most stable top gene for winter and spring, respectively. For summer samples, ΔCT revealed 28s as the most stable while HEL was elected by the NormFinder method. BestKeeper results revealed that 18s, ACT, and 28s were the best gene for winter, spring, and summer, respectively. According to geNorm, M values under 1.5 in all analysis (winter, spring,

summer) were indicated as reliable reference genes for the monitoring of seasonal expression patterns of the target gene. Among them, 18s/28s was the most stably expressed in for winter, and summer (M = 0.876, and 0.382, respectively), and TUB and HEL were top two genes for spring (M = 1.058). Based on the RefFinder, the oder of reference gene stability ranking across three seasons were; winter: (28s, HEL, 18s, ACT,TUB, EF1), Spring (TUB, HEL,ACT,18s, 28s, EF1), Summer (28s,HEL, 18s,TUB, ACT, EF1).

The results from geNorm agreed with the BestKeeper in that the 18s and 28S rRNA gene were the best performers for all tested factors, followed by the 18S rRNA gene and the EF1 gene was the least. However, some inconsistency of RGs was observed among the three seasons. Therefore, the adoption of a specific combination of reference genes is recommended for each of the three sample sets (winter, spring, summer).

4. DISCUSSION

This study is part of our ongoing investigation of the transcriptional changes of Mediterranean mussels in their natural settings. Here, we investigated the transcript levels of common reference genes in the digestive gland of mussels collected from four locations in the Northwestern Adriatic Sea and evaluated their expression stability using four different algorithms (ΔCT, geNorm, NormFinder, and Best Keeper). The sampling strategy was executed to have a different factor such as location, season, and gender to analyze the most viable and robust strategy for accurate qPCR data normalization. The studied genes have been commonly used for qPCR data normalization in different tissues of bivalves in field studies (Cubero-Leon et al., 2012; Lacroix et al., 2014; Louis et al., 2020; Moreira et al., 2015; Soares-souza, 2017).

The four algorithms gave relatively consistent results, with 18s, 28s, and TUB being the most stable RGs across all studied factors, but in different ranking order. The results from the application of geNorm agreed with the BestKeeper in that the 18S rRNA gene was the best

performer under each of the tested factors, followed by the 28S rRNA gene and the EF1 gene was the least suitable.

Therefore, it appeared that when using qPCR to analyze the digestive gland of Mediterranean mussels, the ribosomal RNA genes are more applicable to be used as a reference gene than ACT, HEL, or EF1. A similar is reported for *M. edulis* (Banni et al., 2015; Boonphakdee et al., 2019; Diaz de Cerio et al., 2013; Ruiz et al., 2012). Other than the digestive gland, the highest stability of the 18s gene was observed in gonads, and its stability values appeared not influenced by reproductive stages, (Cubero-Leon et al., 2012), it has previously been used as reference genes in qPCR at different gametogenesis stages, geographical locations, season (Carella et al., 2018; Cubero-Leon et al., 2010; Sara Buratti et al., 2011). Similarly, 28s rRNA is also one of the most stable reference genes in bivalves (Cubero-Leon et al., 2012; Koutsogiannaki et al., 2014; Lacroix et al., 2014).

Other than ribosomal genes, TUB is utilized as an internal control in many studies, including mussels (Lacroix et al., 2014, 2017; Paul-Pont et al., 2016a), snails (Horak and Assef, 2017), clams (Moreira et al., 2015; Review there in Volland et al., 2017). However, there are shreds of evidence that TUB has low stability value among the tissues (Feng et al., 2013) and it may be affected by pollutant exposure (Faggio et al., 2018; Feng et al., 2013).

All investigated genes in this study are involved in the protein synthesis and cytoskeleton structure (Lacroix et al., 2014), and exhibited different stability status in spring season in comparison to winter and summer, suggesting that their expression might be affected by seasonal factors (Cubero-Leon et al., 2012; Banni et al., 2011) (Franzellitti et al., 2020; Picco et al., 2016). A possible explanation for this pattern might be changes of water quality parameters across the three season, for example of chlorophyll –A (food availability) concentration which may have a significant control on the seasonally-changing metabolism of mussels regardless of water temperature (Hatcher et al., 1997). Furthermore, in the sampling area gametogenesis and spawning of Mediterranean mussels takes place in winter and spring, respectively. Those are

major processes in which tissue degeneration and remodeling occurs, likely affecting modulation of some references genes like ACT, HEL (Cubero-Leon et al., 2012; Jarque et al., 2014), while ribosomal genes are maintained stably expressed (Jarque et al., 2014). Moreover, the previous finding that *ACT* expression has a strong correlation with temperature (Jarque et al., 2014), is further confirmed by our results showing instability of this reference genes across seasons. In the Adriatic sea water quality parameters including temperature has subjected to seasonality, (Illuminati et al., 2019; Kraus et al., 2019; Tamburini et al., 2020), and there are evidences of the correlation with physiological responses to temporal variation (Bocchetti and Regoli, 2006; Borković et al., 2005; Petrović et al., 2004; Grbin et al., 2019) .

5. Conclusion

In the present work, we validated two reference genes18s and 28s for qPCR analysis of mussels and, based on the results obtained by the application of from four different algorithms, to be most suitable as reference genes. Combining these two genes appears the best strategy for accurate qPCR data normalization on the overall expression trends of target gene in the digestive gland of the mussels under natural conditions in the field. Although the findings potentially apply to environmental studies related to the seasonal pattern, geographical variation, or gender-based mussel, it is always recommended having a preliminary evaluation of gene stability before attaining the most accurate results. Notably, despite the demonstrated stability of these genes under baseline factors reported here, it would be essential to verify their stability under any experimental conditions directly.

Supplementary data to this article can be found in the Supplement 6

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Chapter 3

General Conclusion and Future Perspectives

The studies performed in this thesis provide novel insights on the molecular mechanisms underpinning the physiological status of Mediterranean mussel (*Mytilus galloprovincialis*) through its sensitive life stages and in response to anthropogenic environmental stressors and under natural fluctuating environmental conditions.

As the first phase of the study, I employed a plastic leachate (styrene monomer) and polystyrene microplastics to understand the modulation of cytoprotective mechanisms during the early embryo stages. The onset of alterations induced by styrene on the morphological, functional (MXR activity), and transcriptional changes have been observed. We showed that the main effects rely on transcripts underpinning physiological functions addressed as potential styrene targets in adult mussels. Moreover, the interactive effects of styrene are challenging for larval development, and impairment of MXR-related transporters and shell biomineralization process might be bottlenecks deciding the bivalve population's vulnerability to environmental changes. To the best of our knowledge, no data on styrene interacting with ABC transporters are currently available for any other marine organisms, suggesting the need for future studies addressing styrene impact on the early stages of other marine species aiming MXR system.

Data reported in the PS-MPs study suggest that the MXR system's modulation may be a part of a generalized response triggered by particle ingestion and stimulation of digestive and immune functions both in the larval and adult stages of mussels. Moreover, our study on PS-MPs revealed the occurrence of physical and transcriptional impairments of genes involved in MXR-related transporters induced by MP, indicating sub-lethal impacts that could increase the larvae and adults' vulnerability toward further environmental stressors. Microplastics are becoming one of the significant environmental threats. Therefore, employing the MXR system observed in this

study is very useful in addressing the possible impacts of MP on other tissues of adult mussels and the other zooplanktonic primary consumers in the future.

Our mussel microbiota study is the first integrative description of the mussel microbiota variation at the tissue scale. According to our findings, mussels exhibit a different microbiota profile compared to the seawater micro-ecosystem, with compositional variations at the organ level. Furthermore, it was emphasized that microbiota localization depends on the mussels' tissue's function and position. Mussels exposed to plentiful contaminants and poorer environmental parameters allow opportunistic pathogens to invade the mussels quickly, ultimately affecting the organism's physiology. Since there is no comprehensive analysis of the whole microbiota profile of each organ of the mussels and their shifting expose to environmental and anthropic stressors, our findings promote future research activities in this area.

Meanwhile, we investigated the stability of common reference genes in the digestive gland of adult mussels, representing different conditions such as location, season, and gender, and we validated 18s and 28s as ideal reference genes for normalization of qPCR data of mussels' represent from a different location, seasons and gender.

Our investigation of transcriptional profiles and microbiota composition of mussels residing in a farm and based on season and gender bias is the first comprehensive description of the physiology of mussels under fluctuating natural environmental conditions. Here, we observed that season- and sex-related differences in expression of different transcripts under the influence of natural environmental variability and gonadal development which set the basis for future investigations on the connection between farmed mussel health and productivity and the environmental quality. These findings provide new insights into the molecular mechanisms underpinning physiology in mussels along with host-microbiome interaction, thus facilitating an integrative approach by combining host transcriptome and microbiome data to unravel the interaction between them in a complex system. Moreover, this approach can potentially be

applied to farmed mussels to monitor their physiological performances o mussels live in different environmental settings.

Comprehensively, this thesis shows how the molecular approach can be favourable employed in assessing different features of mussel physiological responses to environmental stimuli (i.e., pollutant effects on embryo development, changes in feeding efficiency due to microplastic accumulation, adjustment of physiological performances across seasonal changes). Future development of this research activity will be the integration of the molecular data with physiological endpoints and data describing environmental variability within an Adverse Outcome Pathway analysis linking molecular initiator interactions to apical physiological outcomes on growth, shell biogenesis, and reproduction. The ultimate goal is to establish advanced early-warning biomarkers of effect tailored to relevant physiological traits that will support both species conservation and improved productivity, quality, safety, and sustainability of shellfish production under climate change.

Supplements 1: Review

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Microplastic exposure and effects in aquatic organisms: A physiological perspective

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Plastic leachates

ABSTRACT

The impact of microplastics (MPs) on aquatic life, given their ubiquitous presence in the water compartment, represents a growing concern. Consistently, scientific knowledge is advancing rapidly, although evidence on actual adverse effects is still highly fragmented. This paper summarizes the recent literature on MP impacts on aquatic organisms in an attempt to link routes of uptake, possible alterations of physiological processes, and outcomes at different levels of biological organization. Animal feeding strategies and MP biodistribution is discussed, alongside with relevant effects at molecular, cellular, and systemic level. Pathways from animal exposure to apical physiological responses are examined to define the relevance of MPs for animal health, and to point out open questions and research gaps. Emphasis is given to emerging threats posed by leaching of plastic additives, many of which have endocrine disruption potential. The potential role of MPs as substrates for microorganism growth and vehicle for pathogen spreading is also addressed.

1. Introduction

Pollution of aquatic environments by microplastics (MPs), defined as plastic particles of size ≤ 5 mm (UNEP, 2016), originates from the release of primary manufactured particles employed in many industrial and household activities, as well as from the degradation of larger plastics items into micro-sized fragments (Andrady, 2017). The amount of MPs released in the environment is likely to increase as a result of the ongoing growth in the production of plastics worldwide (Plastics Europe, 2018), while degradation is expected over hundreds of years.

The effects of MPs on aquatic organisms are currently the subject of intense research, with an exponential rise in the last few years in

Abbreviations: MP, microplastic; NP, nanoplastic; PE, polyethylene; PET, polyethylene terephthalate; PP, polypropylene; PS, polystyrene; PVC, polyvinyl chloride

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publications reporting data from different taxonomic groups, from laboratory and field investigations, as well as on the role of MPs as vectors for organic contaminants. A Pubmed research on 'Microplastics & Effects & Aquatic' gave 129 hits, with an increase from 6 in 2014–2015 to 58 in 2018; these include at least 20 reviews intended to give a critical perspective on the ecotoxicological consequences of MP ingestion in aquatic biota. In particular, Burns and Boxall (2018) and de Sá et al. (2018) focused on the major knowledge gaps concerning the effects of MPs on aquatic organisms, underlying the mismatch between particle types, size ranges, and concentrations of MPs used in laboratory tests compared to those measured in the environment. Moreover, these Authors addressed the need to understand the mechanisms of action and the capacity of MPs to exert cellular and systemic effects at environmentally relevant concentrations.

This paper will review the most recent literature on the biological impacts of MPs on aquatic organisms Animal feeding strategies and their impacts on the routes of MP uptake and accumulation in different tissues are considered. Moreover, the most relevant effects observed at the molecular, cellular, and systemic level in aquatic species exposed to MPs are discussed.

Where needed, findings related to particles of smaller size (up to 1 μm; Gigault et al. (2018)) classified as nanoplastics (NPs) are also reported as they can provide relevant hints into the biological impacts of small size MPs, particularly in relation to translocation pathways and accumulation at the tissue level.

Overall, building on knowledge gained by previous reviews and meta-analyses (Foley et al., 2018; Galloway and Lewis, 2016; Paul-Pont et al., 2018), this paper attempts to deeply examine relationships between animal exposure, alterations of physiological mechanisms and apical responses, in order to better define the relevance of MP exposure for the health status of aquatic organisms, and to point out currently open questions and research gaps. Finally, emphasis is posed towards emerging biological threats of MP occurrence in aquatic environments, such as leaching of plastic additives, which have the potential to exert adverse effects on aquatic fauna, and the role of MPs as substrate of microorganism growth, which is likely to promote the spread of pathogens as well as the invasion by alien species.

2.1. Uptake mechanisms: indiscriminate feeding vs active selection, and trophic transfer

Given their size range, MPs may fall within the optimal prey range for a variety of aquatic animals. such as zooplankton, mollusks, crustacea, fish, seabirds, and marine mammals, and thus be bioavailable for ingestion (Browne et al., 2008; Cole et al., 2013; Germanov et al., 2018; Lusher et al., 2017). Routes of direct ingestion include particle uptake by filter-, suspension- and deposit- feeders, that have a relatively indiscriminate feeding behaviors by collecting and sorting particulate matter to trap and ingest anything of appropriate size (Moore, 2008). Uptake efficiency also depends on the combination of particle size, shape, and density that determine MP position in the water column and/or sediments, and hence their availability to animals. Typically, low-density polymers like PP and PE float in the water column, while high-density MPs like PS

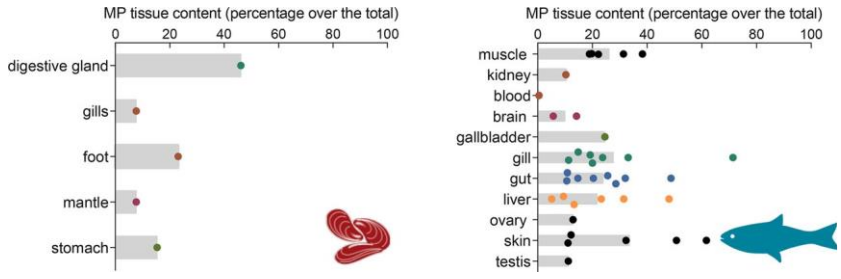

Fig. 1. Resume of the available data on tissue distribution of microplastics in bivalves (left panel) and fish (right panel). Dots represent point data retrieved from the literature and expressed as percentage of the total MP burden reported. Shaded bars are the mean values. Only papers assessing MP concentrations in multiple tissues were considered: Abbasi et al. (2018); Ding et al. (2018); Kashiwada (2006); Kolandhasamy et al. (2018); Lu et al. (2016).

and PVC sink and accumulate into sediments, making them more available to filter/suspension- or deposit- feeders, respectively (Browne et al., 2017; Chubarenko et al., 2016). This issue was addressed in a mesocosm study reproducing PS MP exposure (10 μm; 5, 50 and 250 particle/mL) on a coastal community consisting of dominant invertebrate taxa of the Northern Baltic Sea clearly (Setälä et al., 2016). The results showed that the lowest number of ingested particles was detected in the polychaetes Marenzelleria spp. and Monoporeia affinis, both deposit feeders living either in burrows (Marenzelleria spp) or at the sediment surface (M. affinis). The bivalves Mytilus trossulus and Macoma balthica, filter-feeders at the sedimentwater interface, contained comparatively higher amounts of particles. Uptake of MPs (1, 10 and 90 μm PS spheres at 3–3000 particle/mL) was investigated in freshwater invertebrates with different feeding strategies (Scherer et al., 2017). All species ingested MPs in a concentrationdependent manner with the copepod Daphnia magna, a pelagic filter feeder, consuming up to 6180 particle/h, and exhibiting higher feeding rates on 1 and 10 μm beads compared to the aquatic larvae of the diptera Chironomus riparius and the freshwater worm Lumbriculus variegatus, a burrowing and detritivorous species.

2. Routes of uptake and distribution of microplastics in aquaticorganisms

The above studies suggest that filter feeders are the most prone to ingestion of suspended MPs. These organisms form important links between different trophic levels and between pelagic and benthic ecosystems, hence representing a possible route of trophic transfer for MPs. Recently, the concept that a filter feeding habit may pose a greater risk for MP ingestion was discussed for marine megafauna, in particular to mobulid rays, sharks, and baleen whales (Germanov et al., 2018). These animals have even a higher probability of ingesting relevant amounts of MPs, because they must filter hundreds to thousands of liters of water per day to gain adequate nutrition (Paig-Tran et al., 2013). Therefore, they are likely to ingest MPs both directly from water and indirectly, through contaminated planktonic prey (Dawson et al., 2018; Setälä et al., 2014). Indeed, the estimated daily MP ingestion rates range from a minimum of about 100 pieces for whale sharks from the Gulf of California (Fossi et al., 2017), to thousands of pieces for fin whales inhabiting the Mediterranean Sea (Fossi et al., 2012). This might lead to a significant reduction in their nutritional uptake, as animals feed on the same quantities of particulate matter but receive a lower nutritional benefit (Germanov et al., 2018).

In addition to feeding habits, the mechanism of ingestion and the structure of digestive organs also affect the uptake of MPs. For example, field surveys showed that sea cucumbers (Apostichopus japonicus) select the size of the ingested MPs as long as MPs can fit into their mouth or the tentacles are able to grasp them (Mohsen et al., 2019). Jabeen et al. (2017) showed that in freshwater fish plastic items are likely to accumulate in intestines with more complex coiled structures. Stomachs with a narrow opening to the intestines seemed to retain more plastics. In this light, the results indicate that the presence of complex stomachs and intestines may increase the chances of plastic accumulation and potential damage. The Authors further highlighted that morphological differences in the gastrointestinal tract are related to feeding habits; therefore, the whole structure of the gut and the digestion process should be considered in future investigations on plastic ingestion and effects in fish.

Active selection due to misidentification of MPs for food was also demonstrated. The general idea is that since plastics are not composed of known phagostimulants, their consumption should result from visual or tactile misidentification (Moore, 2008). Visual similarity between plastic debris and preys is suggested to drive plastic ingestion by big aquatic vertebrates such as sea turtles (Schuyler et al. (2014) and reference therein). A similar phenomenon was also observed in small fish or crustacea. For example, omnivorous juveniles of the tropical fish

Girella laevifrons, collected from tidal pools from Las Cruces (Chile), preferentially ingested red colored plastic fibers, since their diet is mainly based on grazing red algae dominating bottom habitats at the sampling locations (Mizraji et al., 2017). Similarly, the planktivorous fish Seriolella violacea preferentially captured black MPs, likely because they appear more similar to food pellets, whereas MPs of other colors (blue, translucent, and yellow) were co-captured only when floating close to food pellets (Ory et al., 2018). In contrast, an example of MP avoidance likely due to visual recognition is provided by a laboratory study in which zebrafish exposed to MPs were able to recognize them as "non-food" and react negatively exhibiting a spitting locomotory behavior (Kim et al., 2019). Zebrafish is equipped with a diurnal-adapted visual system, and it can adjust its movements according to the azimuth of preys (Patterson et al., 2013). In addition, zebrafish displayed good learning and memory abilities, exhibiting long-term behavioral adaptations for judging whether MP was food or not (Kim et al., 2019).

Mysid shrimps (Praunus spp.) are known to actively select their preys (Viherluoto and Viitasalo, 2001a), thus they may discriminate MP fragments from their mesozooplankton preys. Size-selective feeding over MPs of different size classes and irregular shape was observed, since only fragments of a certain size fraction (100–200 μm) were selected over other MP sizes (< 90 μm and > 500 μm) (Lehtiniemi et al., 2018). Particle colors (orange, green, red, and yellow) did not affect selection, in agreement with the finding that mysids locate their prey mainly by mechanoreception (Viherluoto and Viitasalo, 2001b).

Evidence of ingestion due to chemical cues is reported in a wide range of aquatic animals, from cnidarians (Allen et al., 2017), to bivalve mollusks (Bråte et al., 2018) and fish (Savoca et al., 2017). Surface coverage by mixtures of organic and inorganic moleculesis the first modification of surface properties that occurs when MPs are introduced into natural waters, as this brings them into contact with natural colloids, inorganic (ionic compounds and minerals) and organic matter (i.e., mixtures of polysaccharides, proteins, lipids, and nucleic acids), forming a so called "eco- bio- corona" (Canesi et al., 2016; Canesi and Corsi, 2016). Moreover, MPs can be colonized by microorganisms (Galloway et al., 2017), leading to a formation of a biofilm that can contain microbes similar to those feeding in the water column (Vroom et al., 2017), or bacteria-produced exudates acting as infochemicals of food occurrence. For example, DMS (dimethyl sulfide) and its precursor DMSP (dimethylsulfonio propionate) have been identified to drive plastic selection by seabirds and fish (Savoca et al., 2017, 2016). DMS is produced by the enzymatic breakdown of DMSP, which increases during zooplankton grazing (Dacey and Wakeham, 1986), hence it triggers foraging activity for those species whose diet is rich in pelagic crustaceans (DeBose et al., 2008). As a result, preferential ingestion of weathered over virgin MPs was observed in different aquatic organisms (Bråte et al., 2018; Reisser et al., 2014; Vroom et al., 2017). A first evidence of plastic litter ingestion by Pelagia noctiluca, the most abundant jellyfish species in the Mediterranean Sea, was reported by Macali et al. (2018). As with the distribution of plastic litter, the dispersal of this species is mainly driven by local winds and currents that concentrate this organism in regions with a high concentration of floating litter. The presence of PE plastic items in the gastrovascular cavity of the jellyfish was related to active ingestion of the fragments wrongly recognized as food, likely due to chemical cues derived by plastic weathering. It is worth noting that chemoreception is the main sensing mechanism controlling preying and feeding behavior in this species (Morabito et al., 2012). Laboratory experiments with the cnidarian coral Astrangia poculata showed nematocyst discharge and ingestion of different MPs (including PE) (Allen et al., 2017), supporting the hypothesis that phagostimulants potentially found as components of the polymer matrix may promote chemoreception in cnidarians.

Beside the above listed factors that influence mechanical uptake, visual and chemical recognition, some other factors such as shape and hardness also affect the ingestion of MPs. For example, mysid shrimps (Praunus spp.) exposed to primary and secondary MPs within the size range of their prey mostly ingested smaller particles (100–200 μm acrylonitrile butadiene styrene) and spherical primary PS MPs (90 μm), while largest filamentous fragments originated from a soft drink bottle (200–1500 μm polyethylene terephthalate) provoked animal entanglement (Lehtiniemi et al., 2018).

Microplastics may be also ingested indirectly through trophic transfer, whereby contaminated preys are consumed by predators (Au et al., 2017). This issue is the subject of increased scientific debate, in light of the implications of a high seafood diet and related human health effects (Carbery et al., 2018). Laboratory studies gained convincing evidence that trophic transfer does occur for low trophic level organisms. For example, in feeding experiments under controlled, conditions blue mussels (Mytilus edulis) were exposed to fluorescently labelled 0.5 μm PS MPs (at a concentration of about 1,000,000 particle/ mL) before being fed to the common shore crab (Carcinus maenas) (Farrell and Nelson, 2013). The analysis of crab tissues revealed the presence of MPs in the haemolymph, stomach, hepatopancreas, ovary and gills. It must be noted that selected exposure levels of MPs far exceeded those from natural field conditions. A less extreme exposure scenario was employed by Santana et al. (2017). Exposure conditions (MP concentrations, exposure duration, time of post-exposure depuration) were settled to allow MPs (0.1–1.0 μm PVC spherical particles) being detected only in the hemolymph and being absent in the gut cavity of the prey (the mussel Perna perna). Predators were the crab Callinectes ornatus and the pufferfish Spheoeroides greeleyi. PVC MPs were shown to be transferred from prey to predators but with no evidence of tissue accumulation after 10 days of exposure. The Authors hypothesized a reduced likelihood of trophic transfer of particles and, as a consequence, a reduced risk of direct impacts of MPs on higher trophic levels. Trophic transfer of PS NPs was shown in a freshwater food chain with the algae Chlamydomonas reinhardtii, the copepod D. magna, the secondary-consumer Chinese medaka fish (Oryzias sinensis), and the end-consumer Dark chub fish (Zacco temminckii) (Chae et al., 2018). Algae were exposed to 50 mg/L NPs, while higher trophic level organisms were exposed through their diet. Microscopy analyses confirmed that NPs adhered to the surface of the primary producer and were present in the digestive organs of the higher trophic level species. Under these exposure conditions, NPs negatively affected swimming activity of both fish species.

When investigating the fate and biological interactions of MPs in natural settings, it was shown that other factors beside the trophic level can influence the occurrence of MPs in wildlife (Bour et al., 2018a). Nevertheless, many evidences in support of trophic transfer occurring in natural aquatic food webs is accumulating. A first example was reported by Eriksson and Burton (2003) that, through field observations, showed the presence of MPs in the scats of Antarctic fur seals (Arctocephalus tropicalis and A. gazelle) from Macquarie Island (Antarctica). The Authors suggested that MPs had initially been ingested by the plankton-feeding fish, Electrona subaspera, which is the main prey consumed by fur seals in the area. Recently, investigations on stomach contents of wild caught plaices (Pleuronectes platessa) from the Celtic Sea demonstrated an active route of MP trophic transfer from planktonfeeders sand eels (Ammodytes tobianus) (Welden et al., 2018). By analyzing MP content in scats from captive grey seals Halichoerus grypus (residents of a rehabilitation center) and the wild-caught fish they are fed upon (the Atlantic mackerel Scomber scombrus), the study from Nelms et al. (2018) was the first to report empirical evidence for the trophic transfer of MPs to a marine top predator. Further evidence reporting MP occurrence within the gastro-intestinal tracts of various wild-caught fish species (Güven et al., 2017; Lusher et al., 2017) highlight the potential for MP ingestion through feeding on contaminated preys.

The reported findings suggest that trophic transfer represents a potentially relevant route of MP accumulation for any species whose feeding habit involves the consumption of whole prey (i.e. including gastrointestinal tracts), posing direct concerns to human health (EFSA, 2016). Further information on the mechanisms through which MPs transfer through the trophic chain potentially enter the human food chain are advisable.

2.2. *Tissue distribution of microplastics in aquatic organisms*

Generally speaking, MPs are thought to accumulate in aquatic organisms only in tissues in direct contact with water, such as gastrointestinal and respiratory tissues (Grigorakis et al., 2017; Jovanović, 2017; Nicolau et al., 2016). However, as long as scientific evidence is accumulating, it is becoming clear that other tissues may be impacted by particle accumulation (Fig. 1), arguing the onset of translocation processes, and broadening the potential impacts of MPs on a variety of physiological functions.

In bivalve mollusks, gills are a first site of particles uptake, mediated by microvilli activity and endocytosis, while a second route occurred via ciliary movement in the stomach, intestine and digestive tubules, followed by MP translocation towards haemolymph (Browne et al., 2008; Magni et al., 2018; Paul-Pont et al., 2016; Sussarellu et al., 2016; Von Moos et al., 2012). The resulting accumulation pathway shows consistent aggregates within intestinal lumen and digestive tissues, and more limited MP occurrence in gill epithelial cells and in haemolymph

(Avio et al., 2015a; Von Moos et al., 2012). Browne et al. (2008) first showed that in M. edulis particle size influenced the capacity of MPs to translocate from the gut cavity to the hemolymph, with a higher percentage (60%) of smaller particles (3 μm) in the circulatory fluid than larger ones (9.6 μm). However, confocal microscopy demonstrated that both MP sizes were present inside hemocytes. The formation of granulocytomas around accumulated PE MPs up to 80 μm was also observed in the digestive gland of exposed mussels (Von Moos et al., 2012). These findings pointed out the key role of phagocytosis operated by hemocytes in translocation events. Uptake of MPs in multiple organs of mussels under field conditions was recently reported by Kolandhasamy et al. (2018). The abundance of MPs by tissue weight showed significant differences amongst organs, with intestine containing the highest MP levels (9.2 items/g of tissue), surprisingly followed by foot, and then by stomach, gills, mantle, adductor muscle, gonads, and visceral mass. Adherence to soft tissues rather than ingestion was suggested as a possible route of MP accumulation in those organs, such as foot, that are not involved in ingestion processes.

Watts et al. (2014) tested the hypothesis that the non-filter feeding shore crab (Carcinus maenas) can take up MPs (8–10 μm) both through inspiration across the gills and ingestion of pre-exposed food (blue mussels, M. edulis). Gill-mediated inspiration resulted in a 21-days MP body retention, while a 14-days body retention was observed following MP uptake through dietary ingestion. Most MPs were found on the external surface of gills following waterborne exposure or were retained in the foregut, due to adherence to the hair-like setae, after dietary exposure. Jeong et al. (2017) showed that the fluorescence signal of labeled 50 nm PS NPs was dispersed throughout the body in exposed the marine copepod Paracyclopina nana, while florescence of 0.5 μm and 6 μm size PS MPs was mostly restricted to digestive organs. The dispersed fluorescence observed in 50 nm NP-exposed Physiological impacts of microplastic P. nana could be explained by translocation of PS particles across cellular membranes through the digestive organs.

In fish, the gastrointestinal tract is the most investigated organ in relation to accumulation of plastic litter, providing a means to derive information on presence, spatial distribution, and typology of litter in defined areas, or to assess MP body burden in species of commercial interest (Avio et al., 2015b; Pellini et al., 2018; Wieczorek et al., 2018). For

example, digestive tracts of 263 individuals from 26 species of commercial fish caught off the Atlantic coasts of Portugal were examined for content of MPs (Neves et al., 2015a). These were found in 17 species (about 20% of the total). Of all the fish that ingested MPs, 63.5% was benthic and 36.5% pelagic species. Fibers dominated over irregular fragments, with pelagic fish ingesting more particles and benthic fish ingesting more fibers, in relation to the presence of high quantities of fibers in seabed sediments (Woodall et al., 2014). Fish with the highest number of MPs were from the mouth of the Tagus River, in relation to the presence of higher concentrations of bottom marine litter at this location (Neves et al., 2015b), and to riverine inputs from the densely populated metropolitan area of Lisbon. The mackerel Scomber japonicus showed the highest average MP ingestion, suggesting its potential as indicator species to monitor trends of ingested litter in marine regions regulated by the Marine Strategy Framework Directive (EU, 2008).

Beside digestive tracts, the liver was investigated for the occurrence of MPs in commercial zooplanktivorous fish collected in the Mediterranean Sea (Gulf of Lions, France), specifically the European anchovy (Engraulis encrasicolus), the European pilchard (Sardina pilchardus), and the Atlantic herring (Clupea harengus) (Collard et al., 2017). The results revealed that MPs, mainly PE, were translocated into the livers of the three clupeids. In anchovy, 80% of livers contained relatively large MPs (124–438 μm), showing a high level of contamination. Two translocation pathways were proposed: (i) large particles found in the liver resulted from the agglomeration of smaller pieces; (ii) particle translocation through the intestinal barrier (Collard et al., 2017). Abbasi et al. (2018) investigated MP occurrence in gastrointestinal tracts, skin, muscle, gills and liver of commercially-relevant demersal and pelagic fish (Cynoglossus abbreviates, Platycephalus indicus, Saurida tumbil, Sillago sihama) in the Musa estuary and at a site in the Persian Gulf. MP occurrence was widespread in all analysed tissues, although larger size particles were found in the gills and gastrointestinal tracts than in other organs. The means of entry of MPs into tissues not involved in digestion was not clarified, but it was suggested to be related to both translocation and adherence. No MPs (> 20 μm) were detected in the livers or muscles of Asian seabass specimen (Lateolabrax maculatus) sampled in coastal and estuary areas of China, while their prevalence in gut and gill were confirmed (Su et al., 2019). By comparing their results with previous investigations (Abbasi et al., 2018; Collard et al., 2017), the Authors postulated that the relatively big size and shape of the fragments limited their translocation from the intestinal tract to the organs through the circulatory system.

Studies on wild fish populations like those described above provided relevant information about routes of exposure, tissue distribution, and residual body burden under natural exposure scenarios; however, confounding factors mostly related to the methodological issues on sample extraction and handling procedures for MP evaluations may occur (Lusher et al., 2017). Laboratory experiments with model fish species and fluorescently labelled MPs allowed the identification of specific routes and factors promoting MP translocation to selected organs as well as those organs/tissues potentially more impacted by MP accumulation (Fig. 1). For example, tissue distribution of MPs in zebrafish was reported to be size-dependent. By monitoring accumulation of PS MPs following a 7-days exposure to 5 μm and 20 μm size particles, Lu et al. (2016) found 5 μm MPs in fish gills, gut, and liver, while 20 μm MPs accumulated only in gills and gut. The Authors suggested the possibility for smaller MPs to be transferred to the liver through the circulatory system. Indeed, Kashiwada (2006) showed that medaka fish (Oryzias latipes) accumulated 39.4 nm PS NPs (10 mg/L waterborne exposure concentration) mainly in gills and intestine, and they were further detected in the brain, testis, liver, and blood; concentrations in blood reached 16.5 ng/mg protein. Brain accumulation of NPs was further observed in the crucian carp (Carassius carassius) fed with PS NPs through a food chain composed by an algae (Scenedesmus sp.), a crustacean copepod

(D. magna) and the carp fish itself (Mattsson et al., 2017), and in the red tilapia (Oreochromis niloticus) exposed to waterborne 0.1 μm PS NPs (Ding et al., 2018). In both species, neurotoxicity was shown, and in the crucian carp brain tissue structural damages and behavioral disorders were also observed.

A first evidence on maternal transfer of NPs in fish is provided by a laboratory study with zebrafish (Pitt et al., 2018b). Adult females and males (F0 generation) were subjected to dietary exposure to fluorescently labelled PS NPs (51 nm) for 7 days and bred to produce the F1 generation (Pitt et al., 2018b). Four F1 groups were generated: control (unexposed females and males), maternal (exposed females), paternal (exposed males), and co-parental (exposed males and females). PS NPs were found in the yolk sac, gastrointestinal tract, liver, and pancreas of the maternally and co-parentally exposed F1 embryos/larvae. These data suggested that PS NPs are maternally transferred to the offspring via accumulation in the eggs of exposed females, probably due to NP interaction with plasma proteins in oocytes. It was also noted that survival and incidence of deformities were not significantly different across groups, although the maternally- and co-parentally exposed larvae exhibited bradycardia, in agreement with a previous waterborne exposure study (Pitt et al., 2018a).

Larval stages of small fish such as medaka and zebrafish have attracted much interest as models for studies on tissue distribution of fluorescently labelled NPs because they have transparent bodies, rapid embryo development and organogenesis. Kashiwada (2006) showed that PS particles (39.4 nm to 42 μm size) were adsorbed to the chorion in eggs of medaka fish (Oryzias latipes), with 474 nm particles showing the highest bioavailability to eggs. Particles of the size of 39.4 nm moved into the yolk and gallbladder during embryonic development.

Veneman et al. (2017) showed limited PS MP spreading within the zebrafish embryo after injection at the blastula stage, while MP injection in the yolk of 2-day old embryos resulted in redistribution of particles throughout the bloodstream, and accumulation in the heart. van Pomeren et al. (2017) investigated how different uptake routes (chorion and dermal exposure, dermal exposure only, oral and dermal exposure) affected biodistribution of PS NPs and MPs (25, 50, 250 and 700 nm) in zebrafish embryos. Particle uptake within the body was observed only following oral exposure, whereas the dermal route resulted in adsorption to the epidermis and gills only. Following ingestion, the particles spread through the body and eventually accumulated in specific organs and tissues, such as the eyes. Particles larger than 50 nm were predominantly adsorbed onto the intestinal tract and outer epidermis. Embryos exposed to particles via both epidermis and intestine showed highest uptake and MP accumulation in the eye, whereas marginal uptake via the chorion and epidermis was observed.

3. Physiological impacts of microplastic in aquatic organisms

There is relatively little consensus regarding the biological impacts of MP pollution. Indeed, while it is clear that large plastic particles (i.e., meso and macroplastics) can cause readily visible effects at the organism level, such as suffocation, entangling, or intestinal blockage (Gregory, 2009), the direct and indirect physiological effects of small size particles (micro- and nano- plastic) on aquatic animals remain elusive. Fig. 2 reports a conceptual scheme resuming known effects of MPs in aquatic species. By following this scheme, we will revise the current literature reporting molecular, cellular effects of MP ingestion that may aid in explaining outcomes observed at the systemic level and up to apical biological endpoints.

3.1. *Molecular and cellular effects*

Biomarker and transcriptional approaches, both "omic" techniques or quantitative real time PCR targeted analyses, are setting the stage of investigations on cellular and molecular mechanisms and sub lethal effects of MPs/NPs on aquatic organisms, revealing main pathways of interaction on immune-related responses, stress responses (including antioxidants), cell signaling, and cell energy homeostasis (Fig. 2).

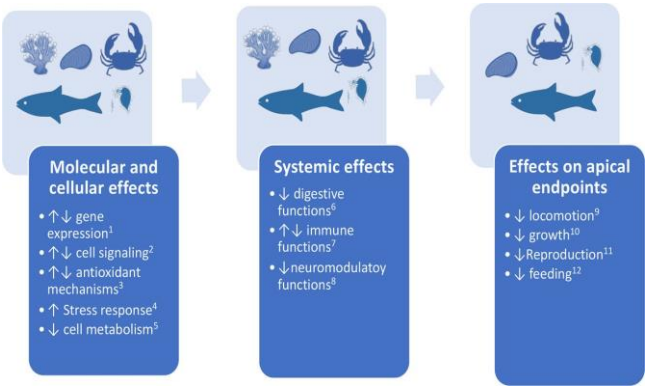

Fig. 2. Resume of the effects at the molecular, cellular, and system levels as well as effects on apical endpoints observed in aquatic organisms interacting with microplastics. [1]LeMoine et al. (2018); Tang et al. (2018); Jeong et al. (2017). [2]Tang et al. (2018); Jeong et al. (2017), 2016. 3 Magni et al. (2018); Tang et al. (2018); Espinosa et al. (2018). 4 5 Jeong et al. (2017); Tang et al. (2018). Bour et al. (2018b); Sussarellu et al. (2016). [6]Capolupo et al. (2018), (Wen et al. (2018b). [7]Avio et al. (2015a), (Paul-Pont et al., 2016), Veneman et al. (2017). [8]LeMoine et al. (2018); Magni et al. (2018). [9]Chae et al. (2018); Mattsson et al. (2017). [10]Critchell and Hoogenboom (2018), Foley et al. (2018); RedondoHasselerharm et al. (2018); Watts et al. (2015). [11]Cole et al. (2015); Foley et al. (2018); Sussarellu et al. (2016); Ziajahromi et al. (2017). [12]Foley et al. (2018); Lizárraga et al. (2017); Murphy and Quinn (2018).

The study from Sussarellu et al. (2016) is amongst the first to infer whole transcriptomic effects of MP exposure in an aquatic organism. Pacific oysters (Crassostrea gigas) were exposed to virgin PS MPs (2 and 6 µm; 0.023 mg/L) for 2 months during a reproductive cycle. Transcriptomic profiling suggested a significant shift of energy allocation toward organism maintenance and structural growth at the expense of reproduction. Indeed, when allowed to spawn, MP-exposed oysters had significant decreases in oocyte number and diameter, and sperm velocity. The yield of D-larvae development of offspring derived from MPexposed parents decreased by 41% and 18%, respectively, compared with control offspring. Imbalance of energy reserves was also reported by Bour et al. (2018b). A 4-week MP exposure experiment (PE MPs, three size classes: 4–6, 20–25, and 125–500 µm, three concentrations: 1, 10, and 25 mg/kg of sediment) with the sediment-dwelling marine clam Ennucula tenuis showed concentration dependent decreases in lipid content and imbalance of total energy reserves.

Using the scleractinian coral Pocillopora damicornis as a model, Tang et al. (2018) reported significant activation of antioxidant enzymes, decreased activity of the detoxifying enzyme glutathione S-transferase and the immune enzyme alkaline phosphatase upon coral exposure to 1 µm PS MPs (50 mg/L, 6 h to 24 h exposure). Supporting these findings, RNA sequencing revealed up-regulation of coral transcripts mostly related to stress response and JNK signaling pathway, while downregulated transcripts were involved in sterol transport and EGF-ERK1/2 signal pathway. These results suggest that acute MP exposure can activate coral stress response while repressing detoxification and immune system likely through the JNK and ERK signaling pathways. Activation of antioxidant responses mediated by the MAPK/JNK pathway was also observed in the monogonont rotifer Brachionus koreanus and in the marine copepod Paracyclopina nana exposed to 0.05, 0.5 or 6µm size PS MPs (Jeong et al., 2017, 2016). Freshwater zebra mussels Dreissena polymorpha exposed to PS MPs (1 and 10 µm, 6-day exposure) showed significant modulation of catalase and glutathione peroxidase activities along with increased concentrations of the neurotransmitter dopamine in circulating fluids (Magni et al., 2018). The Authors postulated an implication of dopamine in mediating the elimination processes of accumulated MPs by increasing cilia movement in the gut epithelium and in gills, in a manner similar to elimination of pseudo-feces, thus MPs being recognized as non-food material. Induction of immune-

related gene products was observed in embryos of zebrafish injected with 700 nm PS MPs (5mg/mL) (Veneman et al., 2017). Notably, RNA sequencing showed enrichment of the complement system, as indicated by up-regulation of transcripts in the alternative complement pathway, which is activated by animal interacting with pathogens not bearing antibodies. Interestingly, up-regulation of immune-related transcripts was observed in early embryo stages of marine mussels (M. galloprovincialis) exposed to 3 μm PS MPs (Capolupo et al., 2018). The uptake activity displayed by embryos towards MPs may have stimulated the immune apparatus, whose function in bivalve early life stages is intrinsically interconnected with the digestive function. Combined analysis of cellular and molecular biomarkers demonstrated that up-regulation of lysosome and immune-related effectors are primary responses to either virgin or contaminated PS MPs in adults of M. galloprovincialis (Avio et al., 2015a; Gaspar et al., 2018; Paul-Pont et al., 2016; Von Moos et al., 2012). Von Moos et al. (2012) showed significant reduction in lysosomal membrane stability paralleled by increased granulocytoma formation in digestive glands of mussels

exposed in vivo to high-density polyethylene (HDPE) MPs (up to 80 μm size). The results suggest a clear sequence of responses: particle ingestion (within 3 h of exposure) is followed by granulocytoma formation (after 6 h) at the tissue level, and finally by lysosomal destabilization at the cellular and subcellular level. Nevertheless, Gaspar et al. (2018) found no significant alterations of lysosomal membrane stability in digestive cells of the Eastern oyster (Crassostrea virginica) after both a 4h in vitro and a 48-h in vivo exposure to 50 nm and 3 μm PS particles. RNA sequencing showed the enrichment of the NOD-like receptor signaling pathway in mussels exposed to MPs (Avio et al., 2015a). NODlike receptors act as intracellular sensors to recognize both pathogenic patterns entering the cell via phagocytosis, and damage-related molecules produced during cellular stress. They operate by activating the non-infectious inflammatory response (Avio et al., 2015a). Furthermore, virgin PS MPs up-regulated various components of the innate immune system, such as putative peptidoglycan recognition proteins. Molecular analyses supported cellular biomarkers also regarding the MP induced modulation of antioxidant defenses, detoxification enzymes, and responses to genotoxic effects (Avio et al., 2015a). The results from Paul-Pont et al. (2016) and Brandts et al. (2018a) corroborated the hypothesis of MPs affecting immune parameters in marine mussels (Mytilus spp.), and further related these effects to increased digestive activities, as some induced immune-related proteins play a dual role in bivalve immunity and digestion of microbial food particles.

LeMoine et al. (2018) investigated the molecular underpinnings of the response to MPs in embryos and larvae of the zebrafish exposed to PE particles (10–45 μm; 5 and 20 mg/L) for up to 14 days. The most noticeable changes are related to down-regulation of transcripts involved in neuronal functioning, neuron differentiation, and axonogenesis, as well as vision related molecules, such as opsin 6 and rhodopsin. Hints towards neurotoxic effects are reported in juveniles of European seabass (Dicentrarchus labrax) (Barboza et al., 2018). MPs caused acetylcholinesterase (AChE) inhibition, increased lipid oxidation (LPO) in brain and muscle, while changing the activities of the energy-related enzymes lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH). Although the mechanisms allowing MPs to affect the neurophysiology of fish are not elucidated, it is worth noting that the reported effects do support previous evidences of MP neurotoxicity in different species (Chen et al., 2017; Oliveira et al., 2013). Furthermore, several metabolic transcripts were consistently down-regulated in MP treatments, in particular those of the glycolytic pathway and of purine metabolism, suggesting profound changes in cellular metabolic pathways (LeMoine et al., 2018). Differential expression of transcripts related to lipid metabolism in the response to MPs/NPs was further corroborated by (Brandts et al., 2018b) in juveniles of sea bass (D. labrax) after a 96 h exposure to ˜45 nm polymethylmethacrylate (PMMA) NPs.

Interestingly, biochemical endpoints also revealed decreased esterase activity levels in plasma and lower levels of alkaline phosphatase in the skin mucus, suggesting that the immune system of fish might be compromised by exposure to NPs.

3.2. *Systemic effects*

A recent study summarized the results form a total of 130 studies reporting ecotoxicological effects of MPs on aquatic organisms (de Sá et al., 2018). Crustaceans were the most commonly studied group (45%), followed by fish (21%), mollusks (18%), annelid worms (7%), echinoderms (7%) and rotifers (2%). These groups occupy different positions in aquatic food webs, with fish generally representing intermediate/top predators that may ingest MPs either directly or feeding on preys containing MPs.

The susceptibility of marine invertebrates towards the physical impacts of MP uptake was first reviewed by Wright et al. (2013), to aid in guiding future research on marine litter and management strategies. The review addressed the factors contributing to the bioavailability of MPs (including size and density), the influence of different feeding strategies, the factors mostly affecting accumulation and translocation, and their trophic transfer through food webs.

Information on the physical impact of MPs in fish has been recently reviewed (Jovanović, 2017), underlying that ingestion of MPs in the gastrointestinal tract of fish can cause different types of histopathological alterations in the intestines. In marine mussels Mytilus spp., exposure to PS MPs (mix of 2 and 6 μm; final concentration: 32 μg/L) caused a number of histopathological alterations (Paul-Pont et al., 2016). In both fish and mussels, histopathological changes were associated with infiltration of immune cells, suggesting inflammatory processes.

In the last few years, the effects of MPs on feeding, growth, reproduction, and survival of freshwater and marine invertebrates have been increasingly investigated: some representative examples are reported.

In the freshwater cnidarian Hydra attenuata, ingestion of MPs in the form of PE flakes extracted from facewash showed significant impacts on feeding (Murphy and Quinn, 2018). The acute and chronic effects of PS fibers and PE beads were investigated in the crustacean Ceriodaphnia dubia (Ziajahromi et al., 2017). PE fibers posed a greater risk than beads in this species, with reduced reproductive output at concentrations within an order of magnitude of reported environmental levels. Environmental concentrations of PE MPs induced harmful effects on development and emergence of the freshwater sediment-dwelling diptera Chironomus tepperi (Ziajahromi et al., 2018). These effects were greatly dependent on particle size, with particles in the size range of 10–27 μm inducing more pronounced responses (Ziajahromi et al., 2018). Chronic exposure of the amphipod Gammarus pulex exposed to PET fragments (10–150 μm size range) did not affect survival, development (molting), metabolism (glycogen, lipid storage) and feeding activity, despite consistent body accumulation at 24 h exposure, which largely depended on dose and life stage (i.e. juveniles ingested more MPs than adults) (Weber et al., 2018).

A number of studies have demonstrated the effects of MPs on zooplankton. Attention was initially focused on marine copepods, a globally abundant class that forms a key trophic link between primary producers and higher trophic-levels. For example, long-term exposure to 20 μm PS MPs alter the feeding capacity and decreased reproductive output of the pelagic copepod Calanus helgolandics, with no significant effects on respiration and survival (Cole et al., 2015). In agreement, prolonged exposure to PET MPs (5–10 μm size; 10,000–80,000 particle/ mL) had negative effects on the reproductive output of the calanoid copepod Parvocalanus crassirostris, which led to decline of the experimental populations (Heindler et al., 2017). In both studies, the reproductive effectswere attributed to decreased egg size and hatching success related to decreased energy intake by impeded feeding due to MP ingestion. In the freshwater crustacean D. magna ingestion of PET textile fibers resulted in increased mortality regardless

of the feeding regime (Jemec et al., 2016). On the other hand, a recent screening study on uptake and effects of four types of environmentally relevant MPs in D. magna and Artemia franciscana concluded that zooplankton crustaceans can ingest various MPs but none of the exposure regimens were highly acutely hazardous to the test species (Kokalj et al., 2018). Other contrasting evidence is provided in macroinvertebrates. Watts et al. (2015) investigated the fate of PP rope microfibers ingested by the crab Carcinus maenas and the consequences for the crab energy budget. In chronic feeding studies, crabs that ingested food containing microfibers (0.3 − 1.0% plastic by weight) showed reduced food consumption; a significant reduction in energy available for growth was observed in crabs fed with 1% plastic. Interestingly, PP microfibers were physically altered by their passage through the foregut and were excreted with a smaller overall size and amalgamated into distinctive balls. In the mud snail (Potamopyrgus antipodarum) exposure to a large range of environmentally relevant polymers did not affect adult morphological and life-history parameters or juvenile development (Imhof and Laforsch, 2016).

Due to the ubiquitous presence of MPs in all aquatic environments, risk assessment for biota has become an urgent research priority that needs standardization. In this light, a recent study evaluated the effect thresholds for a battery of six freshwater benthic macroinvertebrates with different species traits, using a wide range of MP concentrations. Standardized 28-days single species bioassays were performed under environmentally relevant exposure conditions using PS MPs (20 − 500 μm) mixed with sediments (0–40% sediment dry weight) (Redondo-Hasselerharm et al., 2018). MPs did not affect the survival of Gammarus pulex, Hyalella azteca, Asellus aquaticus, Sphaerium corneum, and Tubifex spp. and no effects were found on the reproduction of the worm Lumbriculus variegatus. No effects on growth were found for all species except for G. pulex, that showed a significant reduction in growth. These results indicate that, although the risks of environmentally realistic concentrations of MPs may be low, they still may affect the biodiversity and the functioning of aquatic communities which after all also depend on the sensitive species.

Recent studies focused on the impacts of MPs on development of marine invertebrates. The effects of environmental concentrations of PS MPs were investigated in the sea urchin Paracentrotus lividus and the ascidian Ciona robusta (Messinetti et al., 2018). The feeding strategies of both species proved to be extremely efficient in ingesting MPs. In the presence of microbeads, the metamorphosis of ascidian juveniles was slowed down and development of plutei was altered (Messinetti et al., 2018). Another study on larvae from holothuroids, asteroids and echinoids feeding on 6 μm beads in combination with larger inedible beads showed alterations of the feeding rate (Lizárraga et al., 2017). Despite a difference in sensitivity among species, the clearance rate was decreased at increasing particle number. This study pointed out that MPs, probably perceived as inedible particles, can interfere with normal larval feeding and potentially reduce juvenile quality and performance in nature (Lizárraga et al., 2017). In the marineemussel M. galloprovincialis exposure to 3 μm PS MPs showed sub-lethal impacts on embryo-larval development. However, despite the ingestion and retention of MPs in digestive tract over 192 h, MPs did not impair clearance rate and edible food intake (Capolupo et al., 2018).

Overall, available data in both freshwater and marine invertebrates indicate that differences in uptake and effects of MPs may result not only from variations in the exposure regimes (e.g., duration, particle concentrations), plastic characteristics (e.g., type, size, shape, additives), but also from the species-specific morphological, physiological and behavioral traits, as well as on the life stage investigated. In this light, the environmental physiology of different species should be considered to identify those traits related to enhanced vulnerability towards MP effects.

A meta-analysis of published literature addressed the overall impacts of MPs on

consumption (and feeding), growth, reproduction, and survival of fish and aquatic invertebrates (Foley et al., 2018). Although negative effects were observed for all four categories of responses within different groups, many were neutral, indicating a high variability of responses across taxa. The most consistent effect was a reduction in consumption of natural prey. In particular, this study underlined how those organisms serving as prey to larger predators may be particularly susceptible to the negative impacts of MP exposure, with potential for bottom-up effects through the food web. In this light, it has been hypothesized that MPs may pose a higher risk to larval fish than other aquatic organisms.

A study carried out on larvae from the European sea bass (Dicentrarchus labrax) fed with different concentrations of fluorescent PE MPs of different sizes demonstrated that, although MPs were detected in the gastrointestinal tract of all fish, an efficient elimination from the gut was observed after 48 h depuration (Mazurais et al., 2015). Under these conditions, larval growth and inflammatory responses were not affected, indicating a limited impact on sea bass larvae possibly due to their high potential of egestion. Similarly, no relevant effects of MP ingestion were observed on food consumption and growth of larvae of the fathead minnow (Pimephales promelas) (Malinich et al., 2018). The Authors underlined that fish responses to MPs were highly variable; therefore, care should be taken when interpreting findings of such individual studies.

Finally, recent data indicate that ingestion of MPs can induce behavioral changes in fish. In the jacopever fish (Sebastes schlegelii), 14 days exposure to 15 μm PS MPs decreased foraging time, and gather exposed fish together (Yin et al., 2018). Similar results were obtained for the Amazonian freshwater cichlid Symphysodon aequifasciatus, in which a 30-days exposure to PE MPs (70–88 μm) decreased post exposure predatory performance (Wen et al., 2018a). Several hypotheses have been proposed to explain this behavioral impairment. MPs can disturb the digestion process, cause respiratory stress, or disrupt normal functioning of the nervous system, thus indirectly affect fish general behavior. The effects observed at molecular level on transcription of genes related to neuronal function quoted above underline molecular effects on fish neurophysiology.

These observations draw the attention on further implications of MP ingestion that could deplete the fitness of fish populations in natural environments.

4. The emerging threats of plastic leachates

Following the evidence on the enormous distribution of plastics in the environment, a great body of studies focused on the ingestion, trophic transfer and toxicity of MPs as small physical entities (Wright et al., 2013). Furthermore, attention is addressed to the possible action of plastic particles as vectors for waterborne contaminant dispersion through the environment and their bioavailability to aquatic organisms (Carbery et al., 2018). Indeed, MPs sorb PBT (persistent, bioaccumulative, and toxic substances), such as PAHs, PCBs, DDT, etc., and these compounds can desorb when MPs are ingested by aquatic species (Engler, 2012). Plastic pellets also pick up metals from the water, that can be transported and ingested (Maršić-Lučić et al., 2018). However, some studies pointed out that field investigations provide little evidence that marine MPs affect the global transport of persistent pollutants, and that the ingestion of MPs does not affect bioaccumulation of contaminants in the real environment, despite the evidence from in vitro experiments (Lohmann, 2017). Ingestion of MPs is not likely to increase the risks from exposure to hydrophobic organic chemicals in the aquatic fauna (Koelmans et al., 2016). It is clear that further studies are needed to understand the release processes of MP-sorbed contaminants, their trophic transfer and potential adverse effects. This complex issue has very recently been reviewed (Carbery et al., 2018; Wang et al., 2018; Ziccardi et al., 2016).

In this review, we want to shed the light on the another emerging threat that is posed by

exposure to compounds deliberately added to help the manufacturing process and give the final product properties to be more usable in specific situations or commercially desirable (Hermabessiere et al., 2017). Since these additives are released from plastics in water or may be leached after animal ingestion, they will be henceforth addressed to as plastic leachates.

4.1. Plastic additives and leaching propensity

Plastic products are made from the essential polymer mixed with a complex blend of materials including residual monomers, oligomers, low molecular-weight fragments, catalyst remnants, polymerization solvents, and a wide range of further additives (Hermabessiere et al., 2017). With a few exceptions, additives are not chemically bound to the plastic polymer, resulting in the possibility of migrating within the material and reaching the surface, where they can leach out to the environment (Hermabessiere et al., 2017; OECD, 2009).

About 6000 additives of different chemical classes are used in plastic production. Among them, light stabilisers are added to inhibit the reactions in plastics that would cause chemical degradation after exposure to UV light. Pigments create a particular colour that render the material more desirable. Plasticisers (e.g. phthalates, epoxides, etc.) are added to make plastics softer and more flexible. Antioxidants (e.g. phenols, arylamines, etc.) are utilized to minimize the deterioration caused by heat, light or chemicals that would favour oxygen combination with hydrocarbons. Flame retardants (e.g. halogens and bromine compounds) are added to prevent ignition or spread of flame in plastic material used in electrical and transport applications which have to meet fire safety standards (Thompson et al., 2009).

Bisphenol A (BPA) is used in a number of applications, including epoxy-resin based paints, medical devices, dental sealants, surface coatings, thermal paper commonly used cash receipts (Kang et al., 2003) to make plastics clear and tough. Several studies demonstrated detectable levels in packaged food or beverages (López-Cervantes and Paseiro-Losada, 2003; Vandermeersch et al., 2015). A great amount of studies indicates BPA as an endocrine disruptor (Oehlmann et al., 2009; Rochester, 2013); as such, it has recently been banned from polycarbonate plastics used in baby bottle manufacturing (Brede et al., 2003). Although it is still allowed in the European Union for use in food containers, a temporary Tolerable Daily Intake (t-TDI) of 4μg/kg bw for BPA has been recently established (EFSA, 2015).

Phthalic acid esters, or phthalates, are a group of chemicals widely used as additive in industrial applications. High-molecular weight phthalates, including di(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP), are used as plasticizers in the manufacture of flexible vinyl plastics. The detected concentrations of DEHP and DBP in drinking waters and surface waters are generally below 1 and 10 μg/L respectively (Liu et al., 2017). Nevertheless, phthalates show many effects as endocrine disruptors in animals as in humans, at low concentrations and not entirely through estrogen-mediated pathways (Golshan et al., 2015; Mu et al., 2018; Oehlmann et al., 2009). For example, DEHP (15–30 days of exposure at 10–100 μg/L) reduced sperm production and motility in the goldfish Carassius auratus (Golshan et al., 2015). In the same study, levels of the StAR mRNA, which encodes a regulator of cholesterol transfer to steroidogenesis, and levels of the luteinizing hormone were decreased in DEHP and 17βestradiol (5 μg/L) treated goldfish, indicating interference with fish testis and pituitary hormonal functions. However, DEHP did not alter vitellogenin production and transcription of genes mediating estrogenic effects.

Mu et al. (2018) reported a variety of effects induced by DEHP and dibutyl phthalate (DBP) in zebrafish embryos, including decreased body length, yolk sac abnormities, alteration of immune response, estrogenic effects, and reduced lipid levels. In particular, transcriptomic, proteomic, and lipidomic approaches indicated that the effective

concentration of phthalates required to trigger the immune response, to alter lipid homeostasis and yolk sac development was lower than that required to induce estrogenic effects. This finding points out that other pathways may be more sensitive to phthalates than those estrogenmediated. Indeed, the DBP-induced morphological alterations were not abolished by inhibitors of estrogen receptors, suggesting that disruption of lipid levels may be a possible alternative mediating the phthalateinduced immune response (Mu et al., 2018).

A variety of products, including textiles, thermoplastics used in electronic devices, and products containing polyurethane foams, are added with flame-retardant chemicals to ensure insulation, exclude oxygen, and reduce possible combustion. Flame retardants like polybrominated diphenyl ether (PBDEs) may represent up to 30% by weight of plastic materials (Hermabessiere et al., 2017). Brominated Flame Retardants (BFRs) have been identified in many environmental compartments and also in animals (Guo et al., 2017; Sutton et al., 2019). Differently from compounds of the same class, tetrabromobisphenol-A (TBBPA) is chemically bound to the polymer matrix, so that it has no potential to leach (Morris et al., 2004). No legislation is applied in the European Union to this compound (Vandermeersch et al., 2015). Instead, members of the brominated diphenyl ethers (BDEs) class (i.e. penta- and octa-BDEs) were banned by the European Union since 2004 (EU, 2003), while deca-BDE was banned from electronic and electrical devices since 2009 (European Council Decision, 2009).

Owing to such a variety of plastic additives and their use in high percentage in plastic production, their occurrence in surface and marine waters is not surprising. Additives are found in water and sediments coming from wastewater treatment plan effluents or atmospheric deposition, in rivers and in coastal environments, and a large proportion is leached by the plastic debris when released in the environment (Al-Odaini et al., 2015). Mato et al. (2001) reported 8.9 to 16 μg/g nonylphenols in PP pellets collected in the Bay of Tokyo. High concentrations of PBDEs, BPA and nonylphenols were found in PE and PP fragments collected along beaches and in open sea areas worldwide (Hirai et al., 2011). BPA, PBDEs and 4-nonylphenol were detected in plastic samples found in the Atlantic Ocean (Rochman et al., 2014). BFRs different from TPPBA are not chemically bound to the plastic polymer, and do leach into the surrounding matrix (Engler, 2012; Meeker et al., 2009). PVC, that can contain 10–60% phthalates by weight, is reported to release phthalates into the environment during manufacturing, use, and disposal (Erythropel et al., 2014; Net et al., 2015).

4.2. *Biological impacts of plastic leachates*

The exposure to leachates is underestimated in the literature despite the well-known harmfulness of phenols, phthalates, brominated compounds, etc., which are reported to have adverse effects on the endocrine system of invertebrates and vertebrates, including humans (Canesi and Fabbri, 2015; Correia et al., 2007; Mariana et al., 2016; Sohoni et al., 2001). Chemicals affecting the endocrine system, addressed to as endocrine disruptors (EDCs), act by interacting with specific molecular targets (e.g. hormone receptors, transport proteins, enzymes, etc.) and may produce effects at very low doses. Moreover, depending on the temporal window of the exposure, they may cause long term adverse effects on individuals and their progeny (Alonso-Magdalena et al., 2015). It is also worth noting that EDCs behave differently from most toxicants as they do not always follow the classic pharmacological doseresponse behaviour, and may provoke higher effects at lower concentrations (Vandenberg et al., 2012). These features make investigations on this concern very difficult to perform, and have delayed the definition of regulatory policies. A further challenge is to distinguish contaminants that are sorbed onto the plastics as additives during the industrial manufacturing from those adsorbed from the surrounding medium upon environmental release of plastic fragments

(Hermabessiere et al., 2017; León et al., 2018). Furthermore, for most of plastic products the composition is not fully declared by the manufacturers, so that there is a general absence of knowledge on the potential exposure to additives in the environment or through the diet. On the other hand, additives not only leach from the plastic particles, but they may also be absorbed from the water together with other contaminants and then desorbed (León et al., 2018). Thus, the real biological impact of additives deliberatively included during the plastic production is currently not estimated, although additives constitute a very high percentage of the plastic materials and pose a potentially higher exposure risk (Hermabessiere et al., 2017).

Since MPs are also readily ingested by living organisms and transferred along the trophic chain, leachates may represent an internal source of contaminants released upon intake (Tanaka et al., 2015). A great body of evidence show the occurrence of plastic leachates in body fluids from invertebrates to vertebrates (Poma et al., 2014; Wang and Zhang, 2013). Many in vitro and in vivo studies showed harmful effects of the above classes of compounds and their metabolites (Lithner et al., 2012), including the interaction with different physiological/endocrine pathways (Canesi et al., 2005; Canesi and Fabbri, 2015).

Very few studies combined leaching tests from plastics and toxicity assays of leachates. The potential leaching of additives from a plastic material is due to many factors, including permeability of the polymer matrix, size of gaps between polymer molecules (with the larger favoring the migration), size, solubility and volatility of the additive, pH, temperature, and the chemical/physical properties of the surrounding medium (Kwan and Takada, 2016). The phenomenon also increases with time, and is related to degradation. Lithner et al. (2012) demonstrated that plastics (different classes of polymers) causing acute toxicity (immobilization) to D. magna do leach additives at pH 7 and within a short-term exposure (24–72 h). Leachates were mainly composed by hydrophobic compounds. This work also corroborated previous results from the same group (Lithner et al., 2009) indicating that leachates from PVC were the most toxic. However, no direct analysis of the leachate mixture was carried out, thus no information was given on specific additives; at the same time, only acute effects were investigated Bejgarn et al. (2015) compared the acute toxicity of leachates from 21 commercially distributed plastic items (including DVD cases, phone covers, liquid soap bottles, toothbrush covers, soda bottles) after different periods of simulated weathering (including 0–192 h exposure to artificial sunlight) by assessing their lethal effects on the marine copepod Nitocra spinipes. Changes of pH and smell of the water indicated that new compounds were present in most of the leachates from irradiated objects. Nevertheless, only eight out of the twenty-one objects released leachates with lethal effects on the copepods. The irradiation time seemed an important factor to determine the leachate toxicity. Interestingly, weathering of PVC packaging materials increased the toxicity of leachates, while leachates from PVC cables did not show effects. Although this study applied a wide chemical screening approach, it did not assess single chemical composition of the leachates, so a cause-effect between specific additives and the observed acute toxicity could not be established.

Larvae of the barnacle Amphibalanus amphitrite were exposed to leachate from seven categories of recyclable commercial plastics (Li et al., 2016). Leachates significantly increased mortality, and the toxicity varied according to the type of plastic tested. In particular, the degree of hydrophobicity was positively correlated with mortality, and PVC was found to be the most toxic material, in agreement with previous reports (Lithner et al., 2012, 2009). All plastic leachates significantly inhibited barnacle settlement independently of concentrations. Chemical analyses revealed different complex mixtures of chemicals included in the plastic classes analysed. In particular, LC–MS analysis under the ESI^+ procedure resulted in a minimum of 113 up to a maximum out of 165 peaks corresponding to the different classes of compounds. Analysis under the ESI^- procedure resulted in 5 to 7 peaks. After analysis of the

leachates for specific ESI^+ compounds, only N,Ndiethyl- meta-toluamide (DEET) was returned with a confident spectral match score (> 80%). The Authors concluded that while the leachates were rich in ESI amenable organic substances, those detected are not amongst the commonly monitored organic chemicals (Li et al., 2016).

Leaching experiments with food matrices (e.g. broth, coffee, etc.) widely consumed in expanded polystyrene (EPS) packaging at high temperatures were performed, and leachates used to run toxicity trials on the freshwater crustacean Ceriodaphnia dubia assessed for mortality and reproduction (Thaysen et al., 2018). Some target compounds were expected to be released by EPS but only ethylbenzene was found above the instrumental limit of detection. Leachates affected both biological endpoints, but toxicity did not correlate with ethylbenzene concentrations, suggesting that other substances were released from EPS products that were not targeted by the chemical analyses. Indeed, chemicals which were at concentrations below the limit of detection could have produced cumulative or synergistic effects. Once more, the toxicity of plastic leachate was due to the whole mixtures, mainly of unknown composition.

The toxicity of leachates towards embryo development of brown mussels (Perna perna) was estimated from PP pellets collected in beaches and virgin ones available commercially (Gandara e Silva et al., 2016). Effects of leachates from virgin pellets on embryo development depended on the leaching procedure. The toxicity of leachates from beached pellets completely inhibited embryo development in all samples. The different effects of the leachates from beached and virgin PP pellets was related to the mixture of compounds released: the toxicity of leachate from virgin pellets was likely due to plastic additives only, since the pellets had not been exposed to potential contaminants in situ (Gandara e Silva et al., 2016).

Overall, the above data obtained in invertebrate species, including N. spinipes (Bejgarn et al., 2015), A. amphitrite (Li et al., 2016), D. magna (Lithner et al., 2012, 2009), Lytechinus variegatus (Nobre et al., 2015) and P. perna (Gandara e Silva et al., 2016) indicate that leachates from PVC are the most toxic, while leachates from PE and PP are less toxic or not toxic for certain species. Moreover, leachates from virgin plastics are less toxic than those from plastics collected from the environment.

Since chemical features of the most common additives and their harmfulness are known, a better understanding the extent of their capabilities of leaching and the conditions which favour it is needed. A chemical fingerprint of plastic leachates would also be of interest. However, since the investigations demonstrate that each manufacturer uses different molecules for producing plastic objects, designing a common scheme is almost impossible. Nevertheless, specific studies on the real potential of leachates to impair physiological pathways are strongly advisable. In line with the subtle and specific effects of EDCs, a targeted approach has been carried out by Coffin et al. (2018), who explored the influence of weathering processes due to saltwater, UV radiation, and absorption of hydrophobic organic contaminants from the water on leaching of agonists for estrogen receptors (ER) and aryl hydrocarbon receptors (AhR) into the aquatic medium. in vitro experiments were performed with cell lines (immortalized CYP1A1-blaLS180 and VM7Luc4E2) or larvae of the Japanese medaka (O. latipes) challenged with leachates from virgin and irradiated virgin plastics, and with leachates from plastics recovered from the North Pacific Gyre. Analytical chemistry showed that leachates from the plastic materials were rather different, and contaminant levels in leachates from plastics from the North Pacific Gyre were significantly higher than those from irradiated virgin plastics, suggesting that long-term weathering may increase the ability of estrogenic plasticizers or other adsorbed compounds to be released into water. The biological effects were also rather different. in vitro estradiol-equivalent values for leachates from virgin plastics, UV irradiated virgin plastics and North Pacific gyre recovered plastics were

about 4, 8, and 14 ng/L, respectively. A significant 10-fold vitellogenin induction was observed in medaka larvae exposed to leachates from both North Pacific Gyre recovered and UV-irradiated virgin plastics. in vitro AhR activity was the highest in North Pacific Gyre recovered plastic than in virgin plastic and UV irradiated virgin plastics (toxic equivalency equal to about 1, 0.4 and 0.7 ng/L, respectively. Significant CYP1A mRNA up-regulation (about 18-fold changes) was observed only in larvae of medaka exposed to leachates from North Pacific gyre recovered plastics. It is worth noting that levels of BPA found in the North Pacific gyre recovered and irradiated virgin plastic leachates (0.7–2.6 μg/L and 0.5–1.1 μg/L, respectively) are within the range reported to induce significant adverse effects reproductive system of adult fish (Correia et al., 2007; Liu et al., 2017; Sohoni et al., 2001). Across all plastic leachates, the chemically-based estrogen equivalent concentrations, referred to BPA, OP, nonylphenol and several phthalates, were under-estimated in comparison with the in vitro effects, suggesting the presence of additional compounds which were not targeted or below analytical detection limits. In summary, after 30 days of exposure at UV radiation, leachates from virgin plastic had limited in vitro and in vivo biological activities on ER and AhR. Following irradiation, plastic leachates stimulated AhR activity, which was mostly related to desorbed PCBs. Moreover, in vitro and in vivo estrogenic effects were significantly higher relative to control, and consistent with leaching of BPA and 4-tert-octylphenol. The AhR- and ER- dependent effects of plastic leachates increased significantly with longer exposure in the environment as observed incubating North Pacific Gyre recovered plastic. Although Coffin et al. (2018) suggested that data should be interpreted as a worst-case scenario, the potential for plastics to leach estrogenic additive and be vector of AhR-active pollutants at concentrations causing biological effects clearly emerged.

Overall, most of the different molecules that leach out from plastic materials are often at concentrations lower than analytic detection limits. This reduces the possibility to relate leachate toxicity to a specific chemical fingerprint. The very low concentrations of chemicals do not exclude a cumulative or synergistic toxicity, which in fact was demonstrated by most of toxicity tests on leachates performed so far. It must be stressed that exposure to sub-nanomolar concentrations of phenols and phthalates causes significant effects on aquatic fauna (Balbi et al., 2016; Canesi and Fabbri, 2015) as well as on human cells (Ejaredar et al., 2015; Heindel et al., 2015). Thus, the release of additives, although below the detection limits, has to be considered when investigating the potential biological effects of MPs that could be the sum of mechanical and chemical effects. Also, part of the variability encountered when testing plastic toxicity may derive from the unknown amounts of leachates in the different experimental conditions.

Overall, the paucity of data regarding composition of leachates is limiting comprehensive risk assessment of the environmental impacts of plastic debris in the aquatic environment. The toxicity associated with leachates must be addressed to as a mixture effect that would elude conventional single chemical toxicity assessments. Finally, additive leachates should not be disregarded on the basis of their low concentrations, since most of these compounds do act on animal physiology at sub-nanomolar doses, producing adverse effects in the long term.

5. **Pathogenic bacteria: hitchhikers of microplastics?**

From the early 2000′s, the attention was already turned to the potential of marine plastic debris, that can travel slowly and passively, to promote survival of the associated biota and to transport organisms towards new environments, favoring the invasion by alien species (Barnes, 2002). Zettler et al. (2013) was the first to call ‘Plastisphere’ the environment and community associated to floating plastic debris in the sea. In addition to the meiofauna, the heterogeneity and high surface volume ratio of MPs and filaments offer attractive shelter and create new

ecological niches for bacterial communities (De Tender et al., 2015; Keswani et al., 2016).

Bacterial communities associated with plastic debris are mainly composed by keystone species in biofilm formation, other species degrading MPs and some hitchhikers potentially pathogens (De Tender et al., 2015; Debroas et al., 2017). Main bacterial colonies seem to be generally attracted by MPs as a support rather than by the type of polymer component itself (Oberbeckmann et al., 2016). Biofilms are highly heterogeneous environments and offer several ecological advantages for multitudes of associated bacteria. Biofilm can accumulate nutrients, offers a protective barrier and associated bacteria can organize the degradation of complex substrates (Kirstein et al., 2016). All these features may promote the establishment and growth of diverse types of bacterial communities, including potential pathogens. Recently, a study on coral reefs in the Asia-Pacific region concluded an increased likelihood of disease when corals are in contact with plastic litter (Lamb et al., 2018).

Bacterial communities associated with MPs appear to be different from geographical zones, partially due to abiotic factors (e.g. temperature or salinity) or because of different bacterial composition of the surrounding water (Debroas et al., 2017; Jiang et al., 2018; Kirstein et al., 2016). Moreover, greater bacterial richness was encountered at lower latitudes (Amaral-Zettler et al., 2015). Since the amount of plastic debris may continuously increase, it is expected that the transport and colonization of species towards new environments will increase in the near future.

Due to their long life and low density, MPs can be dispersed by wind and currents from the coast to open ocean over large distances (Cozar et al., 2014). This phenomenon favors the transfer of species from an environment to a new one, allowing the establishment of invasive species. In addition, previous works on microbiome associated to MPs has raised concern about the role of plastic debris as vectors for the dispersal of bacterial pathogens (Keswani et al., 2016; Zettler et al., 2013). In the North Adriatic Sea, the fish pathogenic bacteria Aeromonas salmonicida was detected onto MP fragments (Viršek et al., 2017). This pathogen species, native from temperate waters and higher latitudes, is usually not present in the Mediterranean Sea, and its presence on MP can represent a new source for contamination to fish. The genus Vibrio, that includes many species that are pathogens for humans and marine organisms, has been encountered in several parts of the World as MP-associated communities, with some cases where Vibrio dominated the total abundance of bacteria onto MPs. For example, Kirstein et al. (2016) reported the presence of the human pathogen Vibrio parahaemolyticus on MPs from North and Baltic Seas. Since this strain was also encountered in the surrounding water, the Authors suggested that seawater could serve as a source for MP colonization (Kirstein et al., 2016). Moreover, Vibrio was also present on polypropylene MPs collected in the North Atlantic Ocean and was dominating the total abundance of bacteria associated with the polymer sample (Zettler et al., 2013). The genus Vibrio was also found in 77% of the MPs collected in the Bay of Brest (France) (Frère et al., 2018). In optimal conditions, Vibrio has a fast growth that can explain its occasional dominance on MPs.

MPs can also exert a concentration effect on the distribution of bacteria to living organisms. In most of the cases studied, the bacterial richness was higher on MPs than in the surrounding seawater, demonstrating an attraction effect of bacteria for plastic debris (Bryant et al., 2016; Dussud et al., 2018; Frère et al., 2018). Few pathogens are enough to contaminate the host; hence, if MPs contain pathogenic strains, the number of bacteria entering the organism can be sufficient to cause an infection. Moreover, it is probable that pathogens associated with MPs would be transferred to the food web and persist after passage through the digestive system of the host, where MPs can be enriched in nutrients, this facilitating the growth of other bacterial species. However, studies on the marine mussel M. edulis and the marine worm Arenicola marina showed no alteration of the

microbial composition present on MPs and no enrichment of potential pathogens after the passage through the gut of the animals (Kesy et al., 2017, 2016). Recently, laboratory studies demonstrated that the horizontal gene transfer for antibiotic bacterial resistance was enhanced in MP-associated bacteria communities compared to natural aggregates (AriasAndres et al., 2018).

Overall, available data indicate that MPs can also pose future socioeconomic problems related to spreading invasive bacterial species, transporting fish or human pathogens over long distances, concentrating bacterial uptake by marine organisms or increasing the transfer of antibiotic resistance genes. Moreover, this phenomenon could be potentially accentuated as biofilm participate in forming a protective layer against UV radiation, increasing the life time of plastic fragments. However, research in this domain is only at its infancy and further studies on composition of bacterial communities on several polymer substrates and transfer of genetic elements is required.

6. Conclusions and perspectives

Microplastics represent a global challenge affecting aquatic ecosystems. Given the recent emergence of research in this field, harmonized approaches for the identification and quantification of plastic particles in water and biota are still lacking.

From a risk assessment perspective, it is necessary to develop comprehensive and agreed methodologies to be included in routine biomonitoring programs, especially for the study of exposure to smaller MPs or to NPs. To this concern, the use of mussels as sentinel species for largescale monitoring programs has recently been proposed (Li et al., 2019), and a first attempt to build a Species Sensitivity Distribution (SSD) was proposed as a starting point for possible regulatory guidance (Burns and Boxall, 2018). The SSD was built using ecotoxicity data from marine and freshwater species related to particles of the 10- to 5000mm size fraction (most relevant to environmental size distributions). When compared with the measured environmental concentration (MEC), the resulting effective concentrations show that risks are limited, although some species and life stages appear particularly sensitive (Burns and Boxall, 2018; Foley et al., 2018). However, ecotoxicity approaches do not consider the mechanisms of action and the sublethal effects of MPs that may affect physiology in different model organisms also below SSD derived effective levels. Therefore, knowledge on the effects at the cell/tissue level is necessary, in particular when considering smaller MPs and NPs. Indeed, particles of this size have the potential to pass biological membranes, and consequent implications of adverse effects at the molecular and subcellular levels are of great concern. Moreover, the possible effects of additives have been underestimated so far. Additives occur at high percentage in plastic items, and are reported to leach in the surrounding medium. They are present in leachates at very low concentrations, but those same concentrations are able to affect physiological modulations at the endocrine level (Balbi et al., 2016).

On the whole, this review points out the need for investigations to be performed under a physiological perspective. In this light, evaluating the effects of MPs at the molecular, cellular, tissue/organ and individual level and using multi-system approaches will allow to identify potential common targets of MPs in different species and at different life stages that can be related to changes in selected physiological functions, including immune and stress-related responses, cell signaling, and energy homeostasis. Integrated and multilevel approaches will help to advance the understanding the biological impacts of MPs in aquatic biota.

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Supplement 2: Mini review

Image 1-Indian Beach, Nariman Point, Mumbai. Photo source ©© Shreyans Bhansali

The Silent Killer of the Ocean

By RHGR Wathsala and Silvia Franzellitti

What is a microplastic?

Our daily life has been progressively addicted to utilizing plastic which gives multiple benefits due to its unique characteristics such as versatility, resistant and durability. Have you ever thought that throne grocery bags, water bottles end up in our dinner plates? In reality, this is the truth which is being discovered by scientists nowadays. Plastic waste is ubiquitous in the environment, and it is considered as a geological indicator in the Anthropocene Era (Zalasiewicz et al., 2016).

Though plastic is produced far away from the oceans, ultimately most of the plastic-made litter ends up in the ocean. During the journey from land to the ocean, it is degraded into tiny particles so-called "microplastics" (MPs). The concern on MPs is increased among researchers and society since MPs are recognized as pervasive pollution in the aquatic environment. MPs are defined as plastic particle size < 5mm (UNEP, 2016) that are categorized as primary and secondary based on their production nature. Primary MPs are shed by micro-fragments, beads, fibers, and film which are coming from domestic and industrial effluents, spills and sewage discharges ((Boucher and Friot, 2017). Meanwhile, secondary MPs are generated through the degradation of larger plastic by UV radiation, wave abrasion or microbial degradation (Fig. 4). In facts, primary MPs give a significant contribution to the plastic soup swirling around the World's ocean. Mainly two-thirds of the MPs derive from the synthetic textiles and tires, while the remaining part is made up of microbead presents in cosmetics and personal care products (Boucher and Friot, 2017). In the worst-case scenario, these MPs are small enough to bypass water filtration systems thus, their accumulation in drinking water, saltwater, and aquatic organisms seem unavoidable (Horton et al., 2017).

Microplastics are ubiquitous

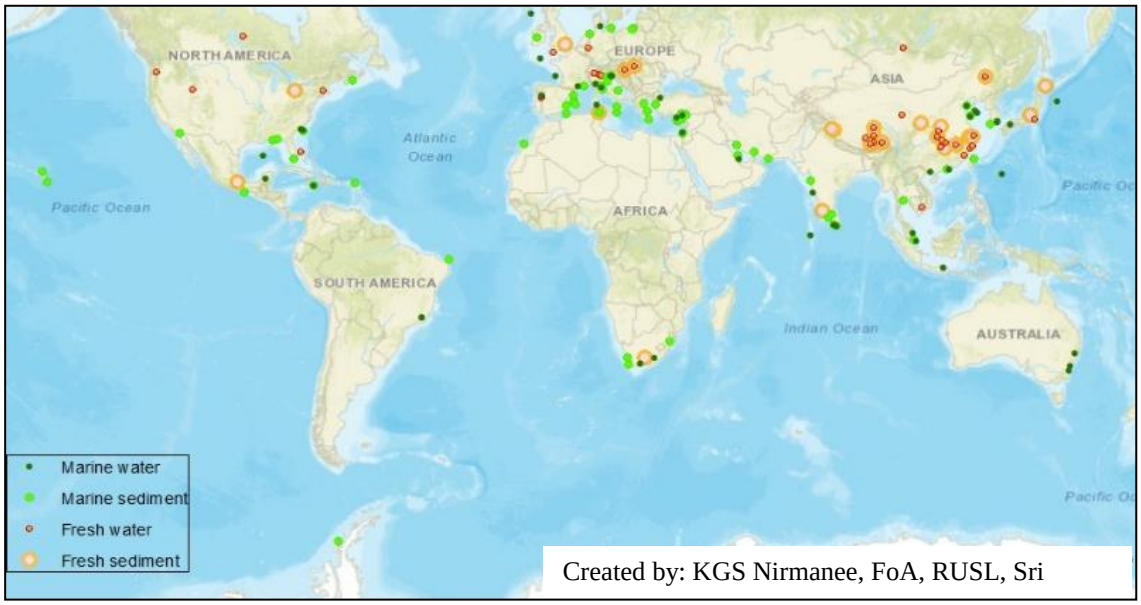


Fig.1. Evidence of MPs presence in marine and freshwater bodies

MP pollution has been reported in marine and freshwater ecosystems and in different environmental matrices,
. The significance of MP contamination in freshwater systems may pose a much great risk to human health because humans are the priority of living being associated with freshwater systems. Therefore, the role of the freshwater system as a major source of MPs to the marine environment should not be neglected and it is needed to equal priority for both ecosystems to avoid MPs accumulation.

Feasting of microplastic

(Based on review paper ;Franzellitti et al., 2019)

Evidence on ingestion of MPs is reported in a range of aquatic organisms, from cnidarians, mollusks, to fish and marine mammals. Basically, MP ingestion by animals depends on the size, shape, concentration and the polymer density that determine the position along the water column. Filter feeders are more vulnerable to ingestion of suspended MPs, especially those made by low- density polymers like polypropylene and polyethylene, which float in the water. Marine megafauna like mobuild rays, sharks, and baleen whales have been prone to accumulate the higher amount of MPs because they have to filter hundreds to thousands of liters of water per day to cover up their food requirement. Hence, they can feel full because their stomach contains full of MPs, but unluckily, they would not able to cover up their food requirements.

Except for filter feeders, most of the other marine organisms mistakenly identified MPs as their food. As an example, omnivorous juveniles of the fish *Girella laeviforms* preferentially ingested red-colored plastic fibers due to their close similarities with red algae on which they graze on. Apparently, the planktivorous fish *Seriolella violacea* attracts black MPs because it is comparable to their food pellet.

Not only the color of the MPs but also chemical cues derived from MPs misled marine organisms. MPs have possibilities to interact with colloids, inorganic and organic matter, forming an eco-corona in the natural environment while it creates bio-corona in the organisms with internal body fluids or secreted biomolecul es. In a laboratory experiment, cnidarians showed that ingestion of different MPs and nematocyst discharges supporting that chemoreception drives to plastic consumption.

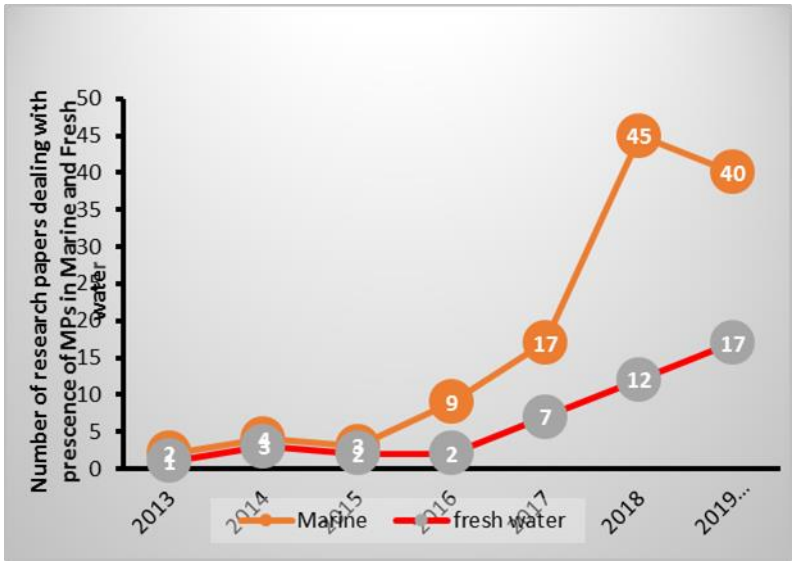


Fig.2. Number of studies dealing with presence of MPs in Marine and Fresh water from 2013 to 2019-May

Whether laboratory experiments are convincing that trophic transfer of MPs does occur for low trophic level organisms, there are many pieces of evidence that MPs can accumulate in top predators like fish species in natural settings. Further, the presence of MPs in the scats of Antarctic fur seals and grey seals highlighted the trophic transfer of MPs (Franzellitti et al., 2019b)

Fears of microplastic (Based on review paper :Franzellitti et al., 2019)

General speaking, MPs accumulation in the digestive tract and respiratory tracts are commonly observed in many aquatic organisms, both invertebrates, and vertebrates. Since researchers are starting to investigate in greater depth of the composition and consequences of MPs on the environment and potentially on aquatic animals, they realized MPs give the significant effect from molecular to the systems level, eventually the whole organism.

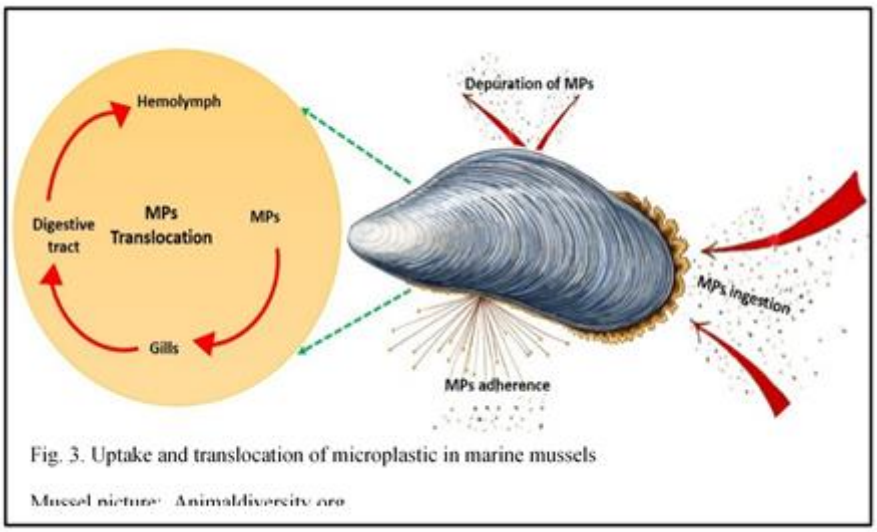
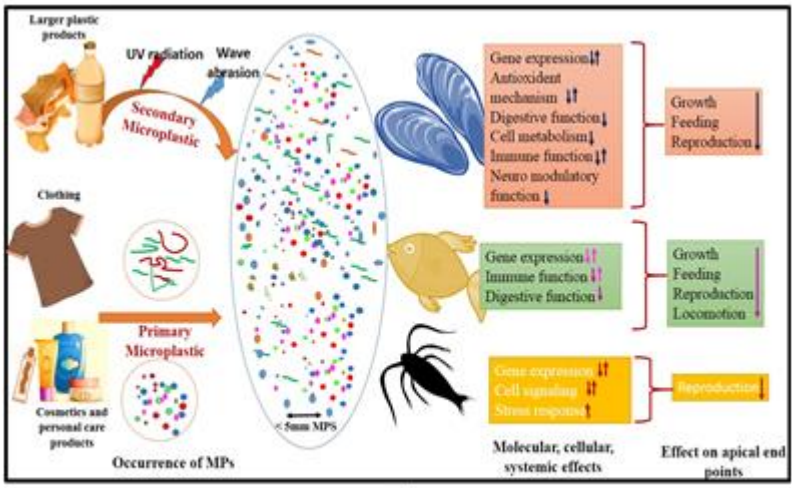


Fig. 3. Uptake and translocation of microplastic in marine mussels

Mussel picture: Animaldiversity.org

Using molecular tools, researchers from Animal and EnvironmentalPhysiology Laboratory, University of Bologna, has identified the transcriptional effect of microplastic at early developmental and adult stages of Mediterranean mussels (*Mytilus galloprovincialis*) ingesting polystyrene MPs. Results of the study published in the journal *Environmental pollution and Comparative Biochemistry and Physiology - C*, have mentioned the fate of microplastic particles in marine organisms.

"The main issue about plastic pollution in marine environments is that micro- to nano-size particles have a size range overlapping with that of food particles ingested by filter-feeders animals, which marine bivalves like mussels, oysters or clams, but also big iconic vertebrates as mobulid rays and whales. The impacts of plastic ingestion include gut blockage, altered feeding and energy allocation, with knock-on effects on widespread physiological processes that are still largely unknown" said, Prof. Silvia Franzellitti, who works on the physiological effect of marine pollutants in aquatic



Biological information have been derived from Franzellitti et al.,

animals, University of Bologna, Italy.

The physiological impact of MPs was witnessed in the copepod. Translocation across the cellular membranes through the digestive tract did not limit MP occurrence to the digestive organs. Besides the digestive tract, in fish, the liver, skin, muscle, gills were identified as possible tissue and organs to accumulate MPs not only due to the action of digestion but also translocation and adherence. Further, studies revealed that polystyrene nanoplastic particles (PS NPs) were accumulated in gills and intestine but also detected in brain, testis, liver, and blood of Medaka fish. It is interesting to note that larval stages of model organisms like zebrafish show the redistribution of polystyrene particles throughout the bloodstream and accumulation in the heart, after injecting MPs in the yolk of 2-day old embryos.

At the molecular levels, the studies on aquatic organisms exposed to MPs pointed out significant impacts on gene expression, cell signaling pathways, antioxidant mechanisms, stress responses, and cell metabolisms likely underpinning dysregulation of digestive functions, immune functions, and neuromodulatory functions (Fig.4). Thus, these research findings ascertained that MPs impair biological system of aquatic animals, ultimately affecting apical endpoints such as feeding, reproduction, growth, and locomotion (Franzellitti et al., 2019b).

Future Perspectives

The MP pollution that we are experiencing today is expected to long-last over the next centuries due to the exponential increase in the use of plastic in modern society and the mismanagement of plastic waste. The examples reported here emphasize the possibilities of transferring MPs to high-trophic level organisms, including human that feed upon whole prey including the digestive tract. Despite the lack of direct evidence on the consequences of MPs to human, available research data are alarming on the possible pernicious effects.

It is unclear the effect and biological transfer of microplastics across all trophic levels from the lowest ones to top consumers. Therefore, researchers from around the world are needed to gather for forming a comprehensive research effort which should comprise organisms from each trophic level of the food web to make a clear picture of this scariest particle.

Though rules and regulations, that are one of the proactive measures for mitigating MP pollution, appear not efficient enough to elucidate the microplastic pollution, it is auspicial gaining a common goal among the whole communities to the smart use and disposal of plastic to the environment.

This article was based on the review article “Franzellitti S, Canesi L, Auguste M, Rasika Wathsala RHG, Fabbri E (2019). Microplastic exposure and effects in aquatic organisms: A physiological perspective. Environmental Toxicology and Pharmacology. 68, 37-51 (https://doi.org/10.1016/j.etap.2019.03.009).

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Supplement 3

The Multixenobiotic resistance system as a possible protective response triggeredby microplastic ingestion in Mediterranean mussels (*Mytilus galloprovincialis*): larvae and adult stages

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Fig S1. Flowchart of the experimental setup in PS-MP treatments with (A) embryos and (B) adults of *M. galloprovincialis*. (A) After oocyte fertilization, embryos were grown in the

presence of PS-MPs at selected nominal concentrations. Changes of MXR efflux activity and *ABCB*/*ABCC* expressions were assessed at the trocophorae (24 h pf) and the D-veliger (48 h pf) stage. **(B)** Adult mussels were exposed for 4 days to 1 and 10 particle/mL PS-MPs at 3 µm or 45 µm size. Changes of MXR efflux activity and *ABCB/ABCC* expressions were assessed in gill biopsies and haemocytes. Accumulation of PS-MPs was also assessed in haemolymph through epifluorescence microscopy. *Ingestion of 3 µm PSMPs by D-veligers at 48 h pf was assessed previously (Capolupo et al., 2018). [a]Fabbri et al. (2014); [b]Capolupo et al. (2018); [c]Franzellitti et al. (2017); [d]Franzellitti et al. (2016); [e]Franzellitti and Fabbri (2013b).

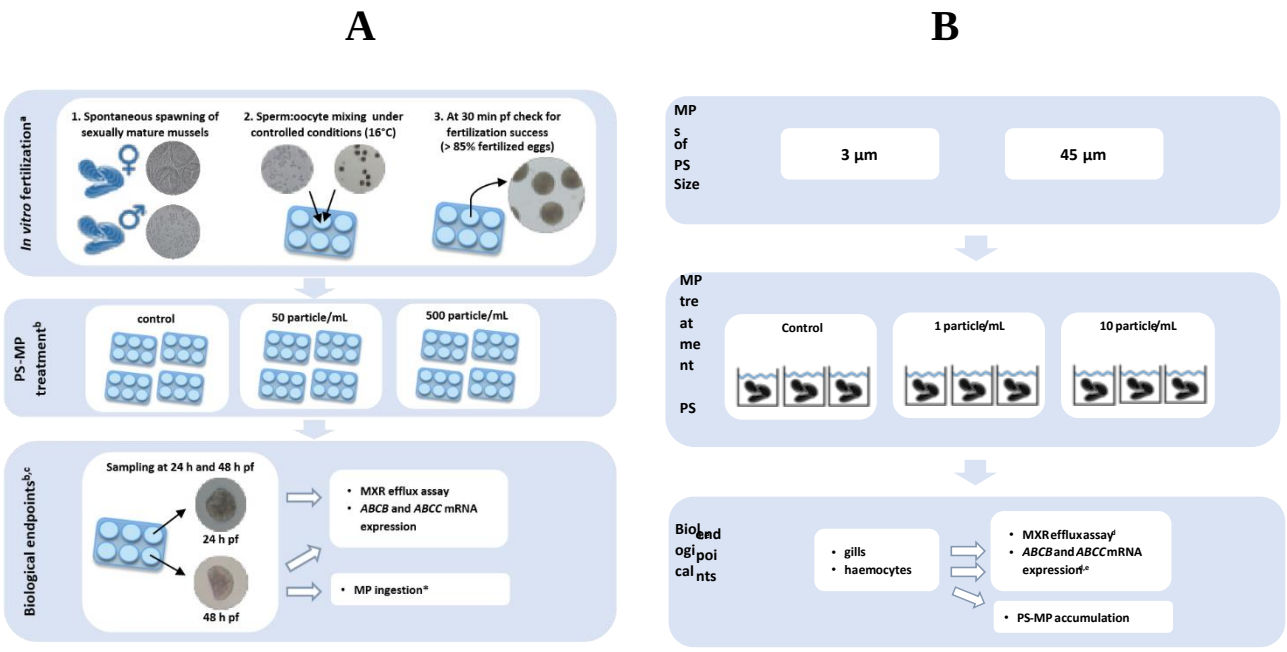

Table S1. PERMANOVA results on the effects of 3 µm PS-MPs ([PS-MP]) on MXR efflux activity and transcriptional responses in mussel embryos at different developmental stages (stage) (998 permutations).

	df	whole dataset		MXR activity		*ABCB*		*ABCC*	
		Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Stage	2	1207.1	0.001	6.07	0.007	451.27	0.001	3711.6	0.001
[PS-MP]	2	58.45	0.001	19.00	0.001	110.32	0.001	9.9248	0.002
Stage x [PS-MP]	4	43.72	0.001	8.92	0.001	87.316	0.001	4.5842	0.004

df = degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P (perm): probability of pseudo-F

Table S2. Pairwise PERMANOVA comparisons for 3 µm PS-MP effects on MXR efflux activity and transcriptional responses in mussel embryos at different developmental stages. Significant values are in bold.

Pairwise comparisons	t	P(perm)	Unique permutations
Trocophorae			
ctr vs 50 part/mL PS-MP	1.3053	0.281	35
ctr vs 50 part/mL PS-MP	2.3978	0.062	35
D-veligers			
ctr vs 50 part/mL PS-MP	**6.7687**	**0.028**	35
ctr vs 50 part/mL PS-MP	**20.77**	**0.026**	35

t: t-statistics; P(perm): probability of t.

Table S3. PERMANOVA results on accumulation of 3 µm and 45 µm PS-MPs in haemolymph (997 unique permutations).

Source	df	Pseudo-F	P(perm)
[PS-MP]	1	6.6943	**0.024**
Size	1	75.398	**0.001**
[PS-MP] x size	1	1.698	0.233

df = degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P (perm): probability of pseudo-F

Table S4. PERMANOVA results on the effects of 3 µm and 45 µm PS-MPs ([PS-MP]) on MXR efflux activity and transcriptional responses in haemocytes and gills of adult mussels (998 permutations).

	df	whole dataset		MXR activity		*ABCB*		*ABCC*	
		Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Tissue	1	428.44	0.001	3948.40	0.001	56.17	0.001	175.58	0.001
[PS-MP]	2	7.75	0.002	2.64	0.119	8.96	0.005	7.45	0.015
Size	1	6.48	0.003	32.53	0.001	7.38	0.01	0.97	0.34
Tissue x [PS-MP]	2	11.29	0.001	5.79	0.026	0.096	0.779	23.39	0.001
Tissue x Size	1	48.10	0.001	1.97	0.182	3.69	0.063	100.45	0.001
[PS-MP] x Size	2	0.67	0.564	0.80	0.382	0.042	0.851	1.27	0.286
Tissue x [PS-MP] x Size	2	39.83	0.001	0.46	0.526	1.26	0.276	85.15	0.001

df = degree of freedom; Pseudo-F: F value by permutation (Anderson et al., 2008); P (perm): probability of pseudo-F

Supplement 4

Tissue-scale microbiota of the Mediterranean mussel (*Mytilus galloprovincialis*) and its relationship with the environment

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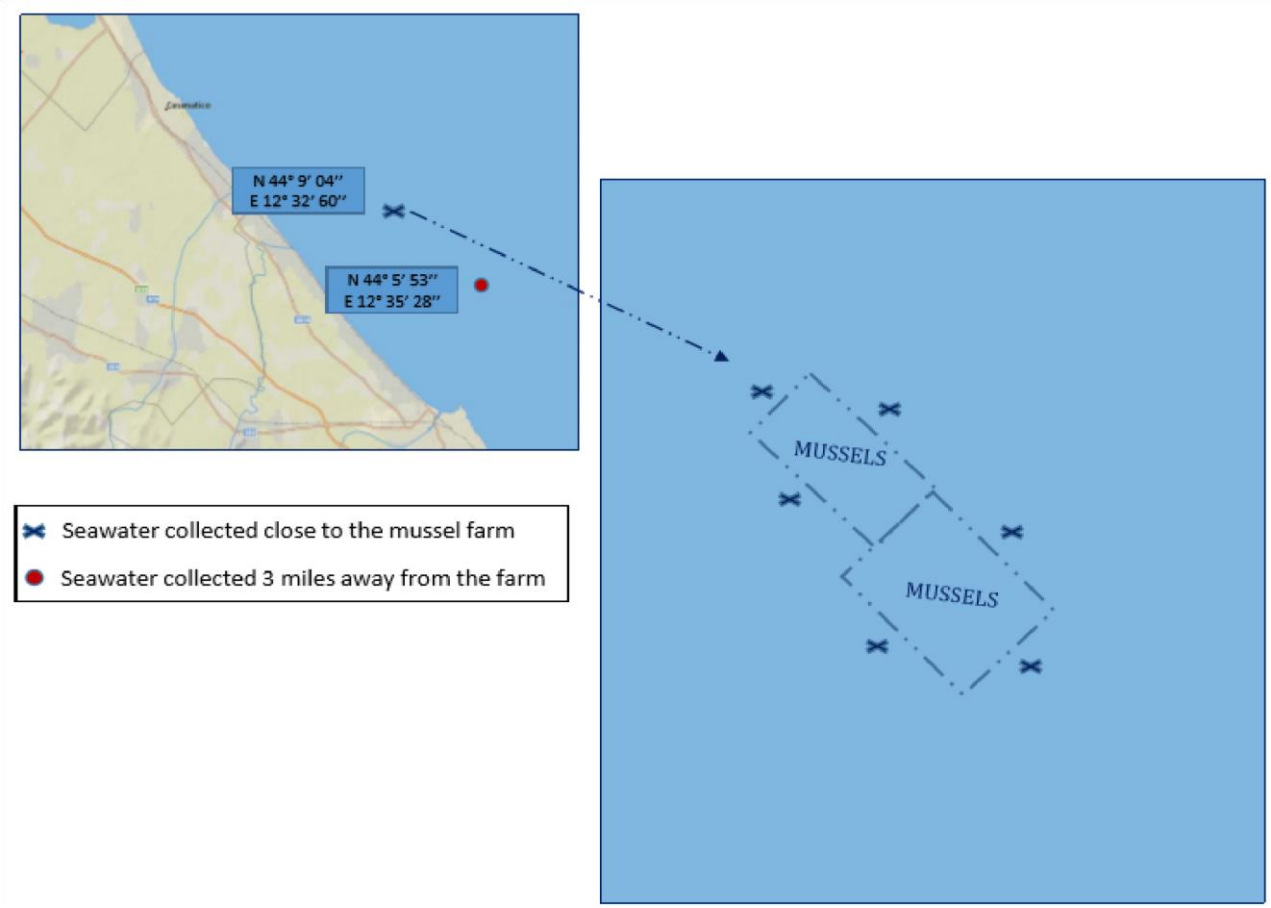
Supplementary Figure S1.

The sampling site of seawater located in North Adriatic Sea coast. Blue cross, seawater collected close to the mussel farm. Red circle, seawater collected 3 miles away from the mussel farm. Samples were collected at a depth of 3 m.



Supplementary Figure S2.

Family-level relative abundance profiles of bacterial communities in the seawater surrounding the mussel farm (FSW) and in seawater taken 3 miles from the farm (CSW). (A). Family-level relative abundance profiles of FSW and CSW. Only families with a relative abundance of ≥1.5% in at least 10% of samples (B). Scatter plots showing the relative abundance values of the main families differently represented between FSW and CSW. The black bar in the graphs indicates the median.

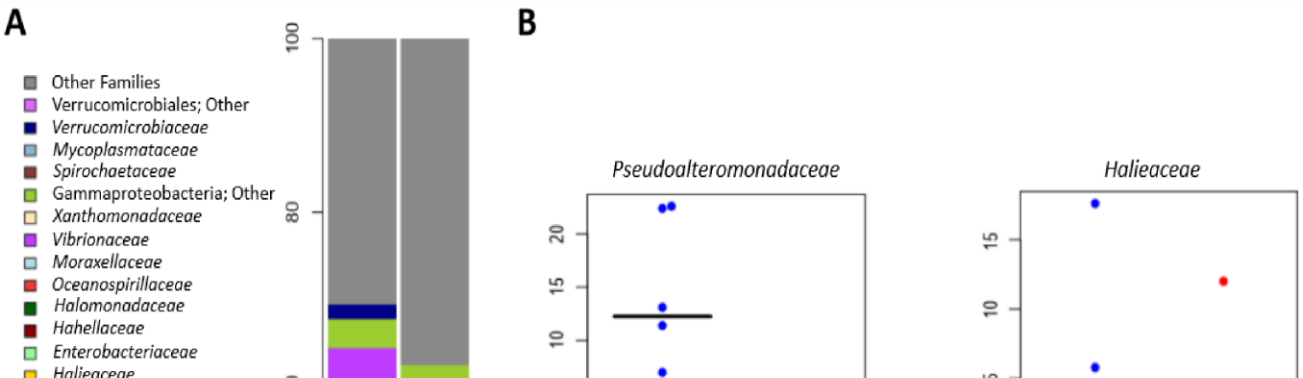


Table S1. Description of the samples. For each tissue type, number of samples, quantity analyzed, and method of extraction are reported. All samples were collected on April 3, 2019.

tissue	samples number	amount analyzed	extraction method
DIGESTIVE GLAND	25	20-30 mg	DNeasy PowerSoil kit
GILL	25	20-30 mg	DNeasy PowerSoil kit
FOOT	25	20-30 mg	DNeasy PowerSoil kit
STOMACH	21	20-30 mg	DNeasy PowerSoil kit

HEMOLYMPH	18	200 µl	DNeasy PowerSoil kit
WATER	7	2 l	DNeasy PowerWater kit

Table S2. Results of adonis statistics applied to the ordination analysis of figure 2 (A).

organs	**R_2**	**P-value**
Digestive gland vs Foot	0.01	0.03
Digestive gland vs Gill	0.01	0.003
Digestive gland vs Hemolymph	0.02	0.002
Digestive gland vs Stomach	0.02	0.002
Foot vs Gill	0.02	0.002
Foot vs Hemolymph	0.02	0.002
Foot vs Stomach	0.02	0.002
Gill vs Hemolymph	0.01	0.006
Gill vs Stomach	0.01	0.03
Stomach vs Hemolymph	0.02	0.002

Table S3. Relative abundance values of the most represented families in *M. galloprovincialis* organs and seawater.

family	**r. a. in digestive gland (%)**	**r. a. in foot (%)**	**r. a. in gill (%)**	**r. a. in stomach (%)**	**r. a. in hemolymph (%)**	**r. a. in seawater (%)**
Bifidobacteriaceae	1.04	2.92	0.09	1.17	0.46	0.00
Microbacteriaceae	0.32	0.13	0.07	1.02	1.02	4.88

Bacteroidaceae	2.06	2.38	0.17	1.34	1.34	0.00
Prevotellaceae	1.69	1.86	0.03	0.83	0.83	0.00
Flavobacteriaceae	7.69	6.93	4.27	19.64	19.64	11.01
Cyanobacteria; FamilyI	0.88	0.04	0.09	1.33	1.33	3.02
Bacillaceae	4.14	0.79	0.68	8.38	8.38	0.00
Lactobacillaceae	1.24	2.11	0.12	0.01	0.01	0.00
Lachnospiraceae	10.42	4.74	1.26	1.70	1.70	0.00
Ruminococcaceae	13.97	5.34	1.55	1.00	1.00	0.03
Erysipelotrichaceae	1.34	0.74	0.43	0.04	0.04	0.00
Veillonellaceae	1.37	0.60	0.11	0.29	0.29	0.00
Planctomycetaceae	3.81	2.67	0.40	0.34	0.34	2.12
Rhizobiales;Other	0.50	0.23	0.21	0.38	0.38	2.70
Rhodobacteraceae	2.89	1.86	1.38	9.43	9.43	8.80
Alphaproteobacteria;Other	1.72	0.14	0.81	0.36	0.36	0.21
Comamonadaceae	2.34	1.56	0.20	0.29	0.29	0.00
Campylobacteraceae	0.07	0.47	1.66	0.80	0.80	2.01
Colwelliaceae	0.34	0.95	0.99	0.88	0.88	0.17
Pseudoalteromonadaceae	0.59	3.27	3.49	0.13	0.13	11.66
Shewanellaceae	0.89	0.77	0.64	0.04	0.04	0.00
Alteromonadales;Other	0.16	0.55	43.43	0.81	0.81	0.01
Halieaceae	1.42	0.16	0.41	4.34	4.34	6.14
Enterobacteriaceae	1.58	0.90	0.41	0.08	0.08	0.00
Hahellaceae	0.08	0.86	11.53	0.84	0.84	0.00
Halomonadaceae	2.18	2.24	0.09	1.49	1.49	0.00
Oceanospirillaceae	0.07	0.51	1.08	0.60	0.60	2.25
Moraxellaceae	1.95	0.97	0.27	0.32	0.32	0.00
Vibrionaceae	2.93	2.18	4.95	1.52	1.52	9.34
Xanthomonadaceae	1.76	1.33	0.22	0.44	0.44	0.00
Gammaproteobacteria;Other	0.36	0.52	1.76	1.17	1.17	3.37
Spirochaetaceae	0.09	22.53	0.26	0.73	0.73	0.00
Mycoplasmataceae	0.39	0.73	0.18	0.07	0.07	0.00
Verrucomicrobiaceae	2.79	2.01	0.96	9.21	9.21	1.63
Verrucomicrobiales;Other	0.70	0.25	0.27	0.79	0.79	0.01
Other Families	24.24	23.73	15.56	28.94	28.94	30.60

Table S4. Over-abundance metagenomic inferred pathways.

pathways	pathways	pathways
1,4-dihydroxy-2-naphthoate biosynthesis I	superpathwayof UDP-N-acetylglucosamine-derived O-blocks biosynthesis	superpathway of glycol metabolism and degradation
1,4-dihydroxy-6-naphthoate biosynthesis I	2-methylcitrate cycle I	mevalonate pathway I
superpathwayof menaquinol-10 biosynthesis	glutaryl-CoA degradation	mono-trans, poly-cis decaprenyl phosphate biosynthesis
superpathwayof menaquinol-6 biosynthesis I	coenzyme M biosynthesis I	mycolyl-arabinogalactan-peptidoglycan complex biosynthesis
superpathwayof demethylmenaquinol-6 biosynthesis I	enterobacterialcommon antigen biosynthesis	mycothiol biosynthesis
superpathwayof demethylmenaquinol-9 biosynthesis	enterobactinbiosynthesis	peptidoglycan biosynthesis IV (Enterococcus faecium)
superpathwayof menaquinol-9 biosynthesis	ethylmalonyl-CoA pathway	peptidoglycan biosynthesis V (β-lactam resistance)
acetylene degradation	formaldehyde assimilation II (RuMP Cycle)	teichoic acid (poly-glycerol) biosynthesis
Bifidobacteriumshunt	formaldehyde oxidation I	NAD salvage pathway II
heterolacticfermentation	fucose degradation	nitrate reduction VI (assimilatory)
hexitolfermentation to lactate, formate, ethanol and acetate	gallate degradation I	octane oxidation
superpathwayof Clostridium acetobutylicumacidogenicfermentation	gallate degradation II	palmitate biosynthesis II (bacteria and plants)
pyruvate fermentation to acetate and lactate II	methylgallate degradation	polymyxin resistance
pyruvate fermentation to acetone	protocatechuate degradation II (ortho-cleavage pathway)	reductive acetyl coenzyme A pathway
pyruvate fermentation to butanoate	nicotinate degradation I	S-methyl-5-thio-α-D-ribose 1-phosphate degradation
allantoindegradation to glyoxylateIII	superpathway of salicylate degradation	sitosterol degradation to androstenedione
creatinine degradation I	toluene degradation IV (aerobic) (via catechol)	starch biosynthesis
androstenedione degradation	glycine betaine degradation I	superpathway of (Kdo)2-lipid A biosynthesis
aromatic biogenic amine degradation (bacteria)	methyl ketone biosynthesis	superpathway of (R,R)-butanediol biosynthesis
aromatic compounds degradation via β-ketoadipate	glycolysis V (Pyrococcus)	superpathway of 2,3-butanediol biosynthesis
4-hydroxyphenylacetate degradation	isoprene biosynthesis II (engineered)	superpathway of geranylgeranyldiphosphate biosynthesis I (via mevalonate)
catechol degradation III (ortho-cleavage pathway)	L-arginine degradation II (AST pathway)	superpathway of methylglyoxal degradation
catechol degradation to β-ketoadipate	L-glutamate and L-glutamine biosynthesis	superpathway of ornithine degradation
biotin biosynthesis II	L-glutamate degradation V (via hydroxyglutarate)	superpathway of sulfolactate degradation
cob(II)yrinatea,c-diamidebiosynthesis I (early cobalt insertion)	L-histidine degradation II	superpathway of sulfur oxidation (Acidianus ambivalens)
cob(II)yrinatea,c-diamidebiosynthesis II (late cobalt incorporation)	superpathway of L-arginine and L-ornithine degradation	superpathway of taurine degradation
adenosylcobalaminbiosynthesis II (late cobalt incorporation)	superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation	thiazolebiosynthesis II (Bacillus)
chondroitin sulfate degradation I (bacterial)	superpathway of L-tryptophan biosynthesis	
lactose and galactose degradation I	L-lysine biosynthesis II	
mannandegradation	L-rhamnose degradation I	
superpathwayof fucoseand rhamnosedegradation	4-deoxy-L-threo-hex-4-enopyranuronate degradation	
1,4-dihydroxy-2-naphthoate biosynthesis I	ketogluconate metabolism	
1,4-dihydroxy-6-naphthoate biosynthesis I	superpathwayof hexitoldegradation (bacteria)	
superpathwayof menaquinol-10 biosynthesis	methanogenesisfrom acetate	
superpathwayof menaquinol-6 biosynthesis I	methylphosphonatedegradation I	

Supplement 5

Variability of metabolic, protective, and antioxidant gene transcriptional profiles and microbiota composition of Mediterranean mussels (*Mytilus galloprovincialis*) farmed in the North Adriatic Sea (Italy)

Table S1. Primers sequences and qPCR performances

Acronym	Transcript name	Primers (5'-3')	Amplicon size (bp)	Amplification efficiency (%)	Accession number	References
Target genes (assessed in digestive glands)						
ABCB	*P-glycoprotein*	CACCATAGCCGAGAACATCC CTCCACGCTCTCCAACTAG	139	112	EF057747	(Franzellitti and Fabbri, 2013b)
Amyl	a-Amylase	CCTCGGGGTAGCTGGTTTTA TCCAAAGTTACGGGCTCCTT	232	90.7	EU336958	(Paul-Pont et al., 2016b)
pk	Pyruvate kinase	AGACTTGGAGCTGCCTTCAG GGAATGCACAGAGGGTTCAT	228	102.33	Locus22823a	(Paul-Pont et al., 2016b)
idp	Isocitrate dehydrogenase (NADP) cytoplasmic	GGAGGTACTGTATTTCGTGAGGC TGATCTCCATAAGCATGACGTCC	104	99.25	Locus2855a	(Paul-Pont et al., 2016b)
lys	Lysozyme	ATGTGGAATCTGAAGGACTTGT CCAGTATCCAATGGTGTTAGGG	368	124	AF334665	(Balbi et al., 2017b)**)**
mt10	10 kDa metallothionein	GGGCGCCGACTGTAAATGTTC- CACGTTGAAGGYCCTGTACACC	346	91	AY566247	**(Dondero et al., 2005)**
mt20	20 kDa metallothionein	TGTGAAAGTGGCTGCGGA GTACAGCCACATCCACACGC	430	92	AY566248	**(Dondero et al., 2005)**
cat	catalase	CGACCAGAGACAACCCACC GCAGTAGTATGCCTGTCCATCC	131	96	AY743716	**(Canesi et al., 2007)**
GST-π	glutathione s-transferase	TCCAGTTAGAGGCCGAGCTGA CTGCACCAGTTGGAAACCGTC	129	100	AF527010	**(Hoarau et al., 2006)**
sod	superoxide	AGCCAATGCAGAGGGAAAAGCAG	177	97	FM177867	**(Koutso**

	dismutase	A CCACAAGCCAGACGACCCCC				**giannaki et al., 2014)**
ctsl	Cathepsin L	CCGAGGCTTCATACCCATATAC CGACAGCGGACATCAAATCT	129	92	AY618311	**(Capolupo et al., 2018)**
hex	Hexosaminidase	GATACTCCAGGACACACTCAATC CTGGTCCATAGCTACCATCAAATA	97	101	EU339934	**(Capolupo et al., 2018)**
gusb	ß-Glucuronidase	GCGGTCATTATCTGGTCTGTAG CCGGTCTTGTTGGGTCTAAAT	112	120	EU339935	**(Capolupo et al., 2018)**
Sex-specific genes (assessed in mantle/gonads)						
VCL	vitelline coat lysin	AGAGCTGTTTTGGCCACAGT TTGCGTTTGACATGGTTGAT	250	100	FM995162	**(Anantharaman and Craft, 2012)**
VERL	vitelline envelope receptor for lysin	CCGAAGGAAATGGAACTGAAA CCCTGCAATCGTATGGAATC	350	100	FM995161	**(Anantharaman and Craft, 2012)**
Reference genes (assessed both in digestive glands and mantle/gonads)						
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)**

Table S2. Results of the BEST/BioEnv analysis

	Best correlated environmental variables	Rho	Level of significance
Whole dataset	• Temperature • Salinity • pH • Chloropyll-a • Gonad maturation level	0.538	0.1 %
Females	• Gonad maturation level • Temperature	0.742	0.1%
Males	• Salinity • Surface oxygen • Transparency	0.846	0.1%

Supplement 6

Reference genes selection for transcriptional analyses in *Mytilus galloprovincialis* under natural field conditions

Gene	Sex	Location	Season
ACT	n.s	n.s	0.0002
TUB	n.s	n.s	0.0006
HEL	n.s	n.s	< 0.0001
EF1	n.s	n.s	< 0.0001
18s	n.s	n.s	0.0049
28s	n.s	n.s	0.0004

Table S1. Significance of CT values of different sampling groups using non-parametric one-way ANOVA (Kruskal-Wallis test) followed by the Mann-Whitney U-test ($p < 0.05$)

n.s =not significant at the 5% level

Table S2. Stability rankings of the candidate reference gene products obtained with different computational methods for male and female samples. Stability values obtained by each method are shown in parenthesis. The gene products are ranked from the most stable (1) to the least stable (6).

Ranking	Comprehensive ranking (RefFinder)	Comparative delta-C_T	BestKeeper	NormFinder	geNorm
Gender					
Male					
1	18s (1.41)	TUB (1.36)	18s(0.974)	TUB (0.621)	18s|28s (0.785)
2	TUB ((1.97)	18s (1.4)	28s (1.137)	18s (0.766)	
3	28s (2.06)	28s (1.42)	EF1 (1.14)	28s (0.825)	TUB (1.16)
4	HEL (4.23)	HEL (1.67)	HEL (1.29)	HEL (1.26)	ACT (1.307)
5	ACT (4.95)	ACT (1.75)	TUB (1.415)	ACT (1.447)	HEL (1.423)
6	EF1 (5.05)	EF1 (1.91)	ACT (1.74)	EF1 (1.629)	EF1 (1.585)
Female					
1	TUB (1.57)	TUB (1.66)	28s(1.44)	TUB (0.643)	18s| 28s (1.034)
2	28s (1.73)	ACT (1.84)	TUB(1.49)	ACT (1.073)	
3	18s (2.63)	28s (1.88)	18s(1.51)	28s (1.234)	TUB (1.447)
4	ACT (2.99)	18s (1.88)	HEL(1.62)	18s (1.312)	ACT (1.532)
5	HEL (4.73)	HEL (2.24)	ACT (2.12)	HEL (1.837)	HEL (1.752)
6	EF1 (6)	EF1 (2.49)	EF1 (2.49)	EF1 (2.156)	EF1 (2)

Table S3. Stability rankings of the candidate reference gene products obtained with different computational methods for different seasons. Stability values obtained by each method are shown in parenthesis. The gene products are ranked from the most stable (1) to the least stable (6).

Season					
Winter	Comprehensive ranking(RefFinder)	Comparative delta-CT	BestKeeper	NormFinder	geNorm
1	28s(1.68)	HEL(1.52)	18s(1.73)	HEL(0.318)	18s|28s (0.876)
2	HEL(1.86)	28s(1.53)	28s(1.8)	28s(0.788)	
3	18s(2.11)	ACT(1.74)	TUB(1.82)	ACT(1.083)	HEL(1.087)
4	ACT(3.66)	18s(1.75)	HEL(1.92)	TUB(1.153)	ACT(1.286)
5	TUB(4.16)	TUB(1.76)	ACT(2.04)	18s(1.237)	TUB(1.355)
6	EF1(06)	EF1(2.89)	EF1(2.5)	EF1(2.73)	EF1(1.865)
Spring					
1	TUB(1.19)	TUB(1.82)	ACT(1.19)	TUB(0.458)	TUB|HEL (1.058)
2	HEL(02)	HEL(2.04)	TUB(1.34)	HEL(1.198)	
3	ACT(2.28)	ACT(2.06)	18s(1.62)	ACT(1.236)	ACT(1.325)
4	18s(3.72)	18s(2.30)	HEL(1.97)	18s(1.744)	18s(1.845)
5	28s(5.23)	28s(2.64)	28s(2.03)	EF1(2.318)	28s(2.046)
6	EF1(5.73)	EF1(2.67)	EF1(2.05)	28s(2.339)	EF1(2.254)
Summer					
1	28s(1.19)	28s(0.94)	28s(0.43)	HEL(0.542)	18s|28s (0.382)
2	HEL(2.06)	HEL(0.97)	18s(0.61)	28s(0.594)	
3	18s(2.06)	18s(0.98)	HEL(0.69)	18s(0.64)	HEL(0.711)
4	TUB(04)	TUB(1.03)	TUB(0.74)	TUB(0.668)	TUB(0.802)
5	ACT(05)	ACT(1.23)	ACT(1.06)	ACT(0.983)	ACT(0.96)
6	EF1(06)	EF1(1.3)	EF1(1.26)	EF1(1.099)	EF1(1.075)

Table S4. Stability rankings of the candidate reference gene products obtained with different computational methods for different locations. Stability values obtained by each method are shown in parenthesis. The gene products are ranked from the most stable (1) to the least stable (6).

Location					
Goro	Comprehensive ranking (RefFinder)	Comparative delta-CT	BestKeeper	NormFinder	geNorm
1	28s(1.19)	28s (1.70)	28s (1.27)	ACT(0.631)	18s|28s (0.829)
2	ACT(2.38)	ACT (1.78)	TUB(1.5)	28s(0.908)	
3	TUB(2.91)	TUB (1.91)	18s(1.57)	HEL(1.104)	TUB1.172
4	18s(2.94)	HEL (2.02)	ACT(1.76)	TUB(1.159)	ACT1.318
5	HEL(4.16)	18s (2.04)	HEL(1.77)	18s(1.585)	HEL1.535
6	EF1(6)	EF1 (3.32)	EF1(3.3)	EF1(3.154)	EF12.129
Catiolica					
1	TUB(1.73)	TUB(1.88)	18s(0.61)	TUB1.181	18s|28s (0.869)
2	18s(1.86)	28s(1.90)	28s(0.79)	ACT1.203	
3	28s(02)	ACT(1.90)	TUB(1.67)	18s1.337	TUB1.388
4	ACT(3.13)	18s(1.92)	ACT(1.69)	28s1.392	ACT1.523
5	EF1(05)	EF1(2.02)	EF1(2.17)	EF11.459	EF11.805
6	HEL(06)	HEL(2.39)	HEL(2.21)	HEL2.089	HEL2.001
Senigallia					
1	TUB(1.68)	TUB(1.48)	ACT(1.86)	TUB(0.783)	18s|28s (0.927)
2	18s(1.86)	18s(1.49)	TUB(1.87)	18s(0.792)	
3	28s(3.34)	HEL(1.71)	18s(2.19)	HEL(1.211)	HEL(1.316)
4	HEL(3.57)	EF1(1.82)	EF1(2.51)	EF1(1.386)	TUB(1.503)
5	ACT(3.83)	28s(1.84)	28s(2.83)	28s(1.504)	EF1(1.624)
6	EF1(4.23)	ACT(1.85)	HEL(2.96)	ACT(1.53)	ACT(1.7)