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Evaluation of tissue morphology and gene expression as biomarkers of pollution in mussel *Mytilus galloprovincialis* caging experiment

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ABSTRACT

The ecosystem is being anthropogenically disturbed, which has serious consequences for the environment and human health, having strong social and economic impacts on the community. One of the most common methods to evaluate the effects of toxic contaminants is based on biomonitoring, e.g., placing *Mytilus galloprovincialis* in the polluted areas investigated. In this study, we have combined two different methods, transcriptomic and morphological analysis, with the purpose of determining whether cell morphology and the ultrastructural organization of our animal model are related to gene expression in outdoor experiments. The most pronounced changes were observed in mussel gills and digestive gland for mRNA involved in protein machinery (*18S*, *28S* and *EF1*), while *HSP70, MT10, CYP4Y1, SOD1*, and *CAT* mRNAs showed scattered modifications not related to the studied area. In agreement with *18S, 28S*, and *EF1* mRNA evaluation, optical and electron microscopy demonstrated an initial inflammatory response of the cells that can lead to apoptosis in the caged mussels in all the polluted areas. In conclusion, the application of a multi-disciplinary approach proved to be effective for assessing the biological effects of contaminations on the health of aquatic organisms, and thus suitable to be applied in eco-toxicological studies. Although affected by several uncontrolled environmental variables, the assessment of mRNA can represent a useful endpoint for an integrated estimation of the overall threats to the sea environment within a field research approach.

1. Introduction

Development of industrial activities and exploitation of natural resources has caused a progressive deterioration of the environment, including pollution of coastal marine ecosystems. The anthropogenic disturbance of the ecosystem has serious consequences for the environment and human health, driving strong social and economic impacts on communities (Fasulo et al., 2015). Polluted marine ecosystems usually contain complex contaminant mixtures; in the areas affected by petrochemical contamination, aromatic hydrocarbons, dioxins, and metals are the major components (Lewis and Santos, 2016; De Vivo et al., 2004; Adamo et al., 2002). This scenario suggests a growing need to develop methods for identifying, assessing, and managing risks posed by environmental contaminants, especially in highly industrial zones such as harbours and oil refineries (Mudu et al., 2014). It is also necessary to remember that environmental monitoring and bioremediation of imperilled habitats, and more in generally disturbed sites, are among the main foci of the European strategies for territory management (Horizon 2020: The EU Framework Programme for Research and Innovation).

One of the most common methods for evaluating the effects of toxic contaminants, e.g., heavy metals and hydrocarbons, is based on biomonitoring, i.e. placing organisms, which have measurable properties that can be used as early warning systems, in polluted environments (Dailianis, 2011).

In this context, bivalves, such as the genus *Mytilus*, are a good choice (Gornati et al., 2016a; Cappello et al., 2013). These

Abbreviations: A, Augusta; North B, North Bagnoli; South B, South Bagnoli; B, Brucoli; C.M., Capo Miseno; qPRC, quantitativePCR; Actβ, Beta Actin gene; CAT, catalase gene; EF1, Elongation Factor 1 gene; HSP70, Heat Shock Protein 70 gene; MT10, Metallothionein 10 gene; CYP4Y1, Mytilus galloprovincialis cytochrome P450 gene; 18S, 18S ribosomal RNA gene; 28S, 28S ribosomal RNA gene; SOD1, Superoxide dismutase 1 gene.

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organisms meet the main requirements to be considered as good bio-indicators in monitoring activities: they are widespread in brackish water and sea environments being thus ecologically relevant; they are easy to retrieve with an easy access to the gametes; they have a high sensitivity to many chemicals, are cost-effective and suitable for caging experiments in field sites (Hamza-Chaffai, 2014; Livingstone, 1993). Furthermore, bivalve mollusc toxicity tests are easy to perform in the laboratory and are generally not time consuming – they take only from 24 h to 48 h to obtain the first meaningful results (Cappello et al., 2015; Libralato et al., 2013).

In this project, we investigated the effects of the presence of sediments contaminated by metals (Fe, Co, Cd, Cu, Zn, and Pb) and C10-C50 hydrocarbons in coastal environment on *Mytilus galloprovincialis* morphology and mRNA expression. The mRNAs considered in this work are those encoding for "stress-related proteins" (*HSP70, MT10, CYP4Y1, SOD1* and *CAT*) and those involved in protein machinery (*18S, 28S* and *EF1*) (Coimbra Rola et al., 2012; Quirós et al., 2007; Gornati et al., 2004). The industrial areas chosen as examples of anthropogenically impacted coastal marine environment comprise the so-called "triangle of death": Augusta-Melilli-Priolo (Di Leonardo et al., 2014) in Sicily (Italy) and Bagnoli (Wang et al., 2015; Albanese et al., 2010) in Campania (Italy). Brucoli (Sicily, Italy) and Capo Miseno (Campania, Italy) were chosen as reference sites not affected by heavy metals and petrochemical contamination.

This project aimed to understand whether the "field experiments", accomplished by using caged mussels from a single population (Cappello et al., 2013), can provide valuable feedback about the eco-toxicological effects when the main endpoint is the evaluation of mRNA. In fact, the mRNA level seems to be an excellent tool as it represents the earliest sign of environmental stress (Gornati et al., 2004). Furthermore, real-time PCR (qPRC) is now a low-cost, efficient method with which several target genes can be evaluated simultaneously. Such a goal is impossible to accomplish with enzymatic or immunohistochemical methods (Lacroix et al., 2014). Nonetheless, since mRNA molecules are the first intermediaries of a highly sophisticated cell mechanism, which includes transcriptional, post-transcriptional, translational, and post-translational regulation (Vogel and Marcotte, 2012), gene expression alone is not informative enough to define the effects on protein profile and functions. Moreover, mRNA changes may be transient because of the adaptive processes developed by the mussels exposed to adverse environmental conditions for a long time and they are often not sustained by modification of the protein content (Gornati et al., 2004).

Although the experimental design in field research is affected by several not-easily controlled variables, such as marine environment parameters (salinity and temperature), sea storms and currents (i.e., sediment resuspension frequency and relevance), inability to place and/or retrieve the cages, seasonality, and even theft, results obtained through this approach provide high-quality knowledge of the real impact of the contamination in these areas.

2. Materials and methods

2.1. Study areas

The "Augusta-Melilli-Priolo" (Syracuse, 37°10′ 39.20100″N, 15°12′26.286″E), chosen as a polluted site for this study, has been classified as a "site of national interest" by the Italian Ministry of Environment (Mudu et al., 2014; D.M. 10.01.2000; L. 09.12.1998) due to the high level of pollution and subsequent risk for human health. The other two polluted areas were in the industrial area of Bagnoli (Naples), North pier (40°48.167′N, 14°09.903′E) and South pier (40°48.659N, 14°09.515E), which is included in Italian national legislation for environmental reclamation of disused and heavily polluted coastal sites (Romano et al., 2008). Conversely, Brucoli (Syracuse, 37°17′23.67″N, 15°12′40.68″E) and Capo Miseno (Naples, 40°46.931N, 14°04.515′E) were chosen as reference sites (Fig. 1). Evidence of these contaminations was provided by Laboratorio Ecocontrol Sud (Syracuse, Italy), which analysed sediment samples of Capo Miseno, North and South Bagnoli; whereas for Augusta and Brucoli the University of Messina ascertained the eventual presence of contaminants in these areas (Maisano et al., 2016).

2.2. Experimental design

Adult *Mytilus galloprovincialis* Lamarck, 1819 (5.2 ± 0.4 cm shell length) were obtained from aquaculture Goro angler association (Ferrara-Italy) in the summer season. Mussels were maintained in aerated seawater in large, flow-through holding tanks for 2 weeks and then transferred to Augusta, Brucoli, North Bagnoli, South Bagnoli, and Capo Miseno for 60 days, from October 2013 to December 2013, in stainless steel cages deployed by scuba divers. Temperature values at the depth of the cages varied from 17.9 °C to 15.1 °C in the Sicilian sites and from 21.6 °C to 15.7 °C in the Campania areas over the experimental period. Salinity levels showed no significant variations over the monitoring period, ranging from 37.4 to 37.7 PSU in the sampling sites of Campania while slightly higher values were registered in the Sicilian sites (37.9 ± 1 PSU). Mussels were retrieved; then the gills and digestive gland were rapidly excised from fifteen mussels per site. Tissues were frozen in liquid nitrogen and then stored at −80 °C for molecular analysis. For morphological evaluation tissues were fixed in 1% Karnowsky solution in 0.1 M sodium cacodylate buffer (pH 7.4), for 24 h at 4 °C, and then preserved in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from approximately 100 mg of gill and digestive gland homogenate (n = 10), using the TRIzol solution (Invitrogen™, Italy) according to the manufacturer's instructions. The extracted RNA was quantified by spectrophotometer and its integrity was checked by gel electrophoresis. The first-strand cDNA was synthesized using the IScript cDNA Synthesis Kit (BioRad, Italy) according to the manufacturer's instructions. The generated cDNA was stored at −20 °C.

2.4. Quantitative PCR

qPCR was performed using iTaq™ Universal SYBR® Green Supermix (BioRad, Italy). Specific primers were designed using the Beacon Designer Program (BioRad, Italy) within the sequences of the genes shown in Table 1. *Actβ* was used as housekeeping gene because its expression proved to be more stable than other genes tested (*GAPDH* and *18S*). Each reaction tube was set up according to Rossi et al. (2009) with some modifications. Briefly, 7.5 μl of SYBR Green Supermix (2x), 1 μl of forward and reverse primer (6 μM), 1 μl of cDNA (diluted 1:5), and water to a final volume of 15 μl were mixed and run in the CFX 96 Thermocycler (BioRad, Italy). Thermal cycle was set as follows: 5 min at 95 °C, 10 s at 95 °C and 30 s at 60 °C for 40 cycles. Each experiment was repeated three times.

2.5. Morphological analysis

Fixed specimens were buffer-rinsed, postfixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated in ethanol, and embedded in Epon-Araldite 812 as previously described (Bava et al., 2013). For histological analysis, sections (700 nm thick) were cut and stained with crystal violet-basic fuchsine dye (Bio-Optica,

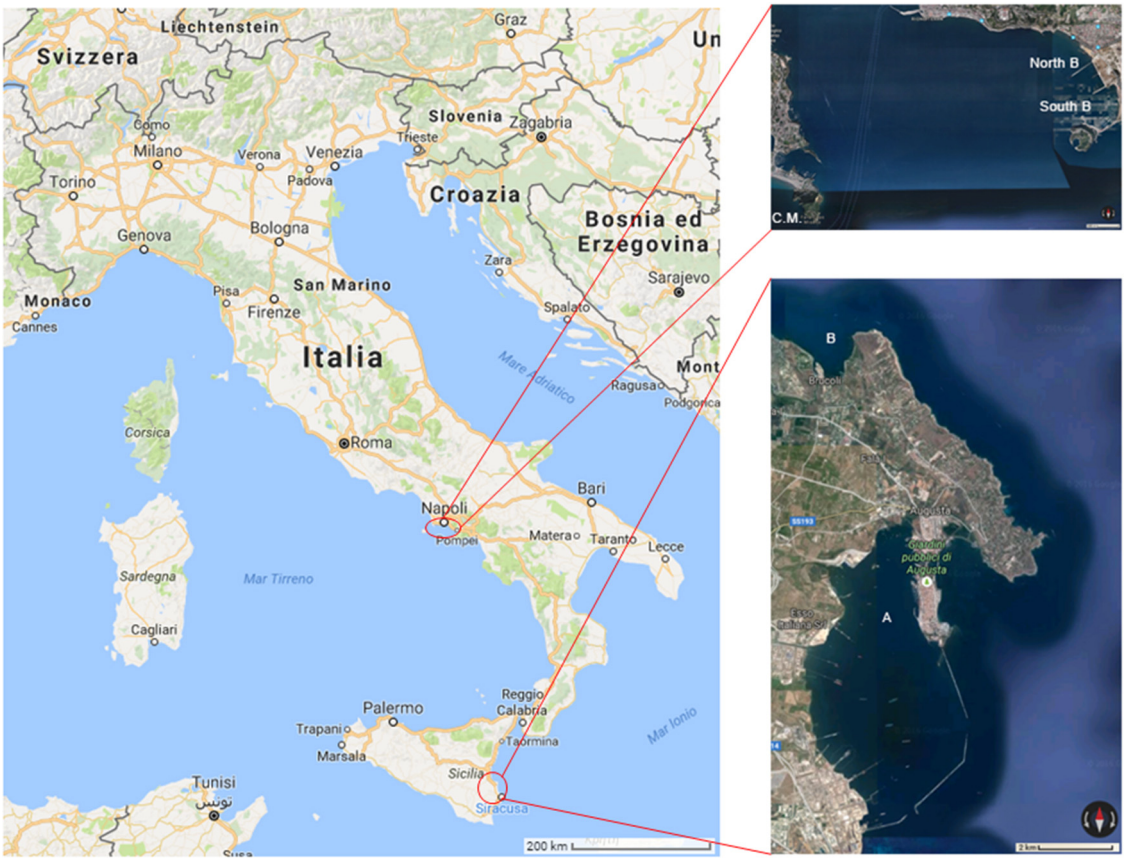

Fig. 1. Map showing the mussel caging sites.

Table 1
Primers used in this work.

Gene Name	Sequence 5′-3′	Amplicon length	Accession Number
Actβ *FW Primer*	GCCCGATGGACAGGTTAT	76	AF157491.1
Rev Primer	CAAGAAGGATGGTTGGAATAATGA		
HSP70 *FW Primer*	ATAACTACTGAGATATGGCAGGAA	115	AJ783713.1
Rev Primer	TGGTCGTTGGCTATGATGT		
MT10 *FW Primer*	AAGATCACTGTGACTACTACGAAT	78	AY566248.1
Rev Primer	TGCCACAGATACACACATTTG		
SOD1 *FW Primer*	GTGACAGTGACAGGAGAGTTA	120	FM177867.1
Rev Primer	TCCAAATGGGTTGAAATGTGAT		
CAT *FW Primer*	CTGTTCTCTGACCGTGGAA	82	AY743716.2
Rev Primer	CCTTGTTGACCGTCTTAAATGT		
CYP4Y1 *FW Primer*	ACCAGTTCCCTTAATTTCAAGAC	111	AF072855
Rev Primer	GGGTTGTGATGGAGACCA		
18S *FW Primer*	CCTGCTTACCTTCCTCCAT	75	JX081670.1
Rev Primer	CCTGCGTGTTATGCTTTGT		
28S *FW Primer*	TGACTCTAGTCCGACTTTGTG	118	AB103129
Rev Primer	CGCTCTCCGCTTAACTGA		
EF1 *FW Primer*	GATTGTGCTGTGTTGATTGTTG	83	AB162021.1
Rev Primer	GCGTGTTCTCTGGTCTGA		

Italy) according to a standard protocol. The specimens were washed with distilled water, dehydrated with ethanol (70%, 90%, 95%, and 100%), then placed in xylene and mounted using Eukitt (Bio-Optica, Italy).

For ultrastructural studies, sections (90 nm thick) were cut using a Pabisch Top-Ultra A ultramicrotome (Emme 3S.r.l., Italy), collected on Cu/Rh 300 mesh grids, counterstained with uranyl acetate and lead citrate, and examined under a Morgagni electron microscope (Philips, Eindhowen, NL) at 80KV.

2.6. Statistical analysis

The Ct values were recorded and the relative gene expression, expressed as $2^{-\Delta\Delta Ct}$ ($\Delta Ct = CtTarget - Ct Act\beta$ and $\Delta\Delta Ct = \Delta CtExposed\ samples - \Delta CtControl\ samples$), was taken as a dependent variable. Data analysis was performed by one-way ANOVA ($p = 0.05$), completed with Scheffé's test ($p < 0.05$) in order to determine which mRNAs were significantly different.

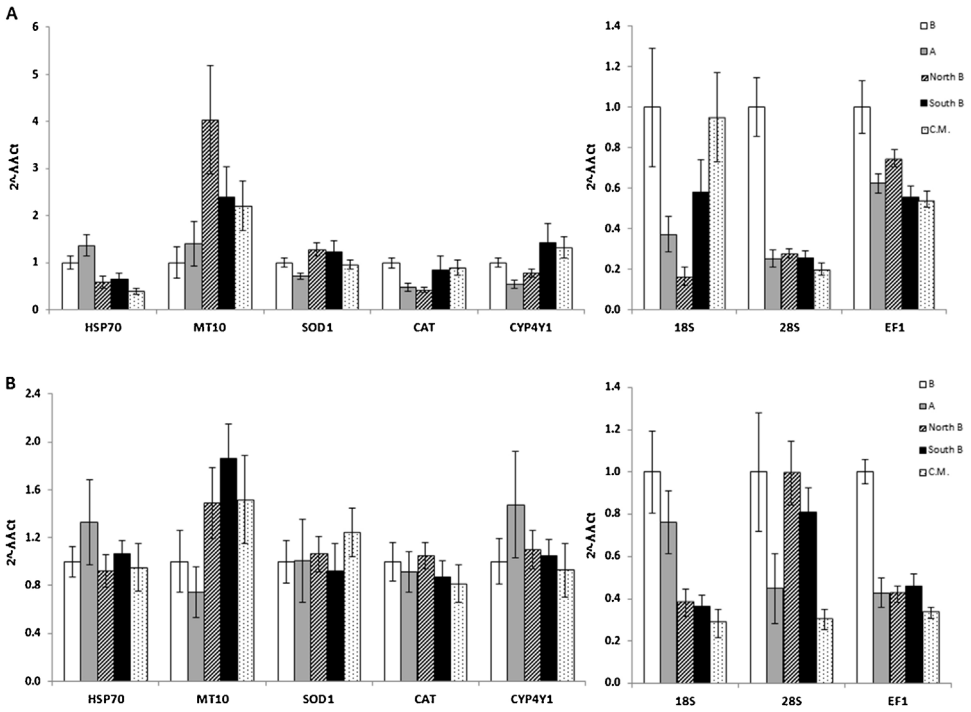

Fig. 2. qPCR of the selected genes. mRNA expression of *HSP70, MT10, SOD1, CAT, CYP4Y1, 18S, 28S*, and *EF1* in gills (A) and digestive gland (B) of mussels caged in the areas considered in this study. The gene expression is reported as fold change compared to Brucoli (white bars), the site considered as control. Values were normalized with the reference gene *Actβ*. *Scheffé's test, $p < 0.05$.

3. Results

3.1. Sediment contamination

High levels of contamination have been well documented by previous research (Mercogliano et al., 2016; Wang et al., 2015; Di Leonardo et al., 2014; Tornero and Ribera d'Alcalà, 2014; Magno et al., 2012; Albanese et al., 2010; Romano et al., 2009, 2008) in the sediments of the investigated areas (Bagnoli, Augusta) and were confirmed by the results obtained through sediment characterization in the present research. Specifically, among the analysed elements and compounds, As (up to 32 mg/kg), Pb (up to 143 mg/kg), and Zn (up to 362 mg/kg) for the Bagnoli areas and Hg (up to 143 mg/kg) for the Augusta bay should be considered of high environmental relevance because they significantly exceed the reference values.

3.2. Real-time PCR

Brucoli was used as reference site to evaluate the results from the contaminated sites.

mRNA expression of the selected panel of genes in gills and digestive gland exhibited different behaviours (Fig. 2A and B). In both tissues, however, the most meaningful changes ($p < 0.05$) were observed for those genes involved in protein machinery (*18S, 28S* and *EF1*) rather than in those belonging to stress response (*HSP70, MT10, SOD1, CAT*, and *CYP4Y1*). A detailed examination indicates a downregulation of *18S, 28S*, and *EF1* mRNAs in all the examined samples, including those of Capo Miseno, when compared to mussels placed in Brucoli, the area that we considered as control. A few exceptions were observed in gill *18S* mRNA of mussels caged in Capo Miseno, and in digestive gland *28S* mRNA of mussels caged in North Bagnoli. As for the mRNA expression of the stress related genes, in gills, the most interesting observations concern the downregulation of *HSP70* and the upregulation of *MT10* and *SOD1*, except for the animals caged in Augusta. In digestive gland, no significant difference ($p > 0.05$) was found regarding mRNA expression except for *CYP4Y1* of mussels caged in South Bagnoli compared to those caged in Augusta. The details of statistical significance are reported in Table 2.

3.3. Morphological analysis

3.3.1. Optical microscopy

Histological observation of gills, sampled after 60 days in the areas of interest, showed relevant tissue modifications, depending on the site (Fig. 3A). In comparison with the control (Fig. 3, Panel Aa), a consistent infiltration of hemocytes was observed in gills of Augusta and Capo Miseno (Fig. 3, Panels Ab and Ac) and become more evident in South Bagnoli (Fig. 3, Panels Ae and Af). Simultaneously, the ciliated epithelium was affected, the structural changes consisting of thinning of gill filaments (GF) and erosion of frontal (FC) and lateral cilia (LC) These impairments were most evident in the mussels caged in South Bagnoli (Fig. 3, Panels Ae and its enlargement Af) and Augusta (Fig. 3, Panel Ab), but also present in some specimens of the samples caged in Capo Miseno (Fig. 3, Panel Ac). Hyperplasia, due to an increased number of cells, was observed in the mussels caged in North Bagnoli (Fig. 3, Panel Ad).

The tissue morphology of the digestive gland (Fig. 3B) showed evidence of general disorganization in the samples from polluted areas compared to those of the control site (Fig. 3, Panel Ba). These tissues were characterized by the loss of consistency, vacuolization of digestive tubules, and massive infiltration of white adipose tissue (Fig. 3, Panels Bc, Bd). Permeation of hemocytes was also observed (Fig. 3, Panels Bb-f). All these findings suggest that both gills and digestive gland are sensitive to water pollution.

3.3.2. Transmission electron microscopy

Epithelium of mussel gills (GE) consists of heterogeneous cell types that include large non-ciliated and ciliated cells (EC), with extended cytoplasm and decondensed nuclear chromatin, small cells (SC) with a high nucleus/cytoplasm ratio, and large gran-

Table 2
p-Values of pair wise comparisons. Values of gene expression of gills are reported above the diagonal. The corresponding values for digestive gland are reported below the diagonal. The set of eight values correspond to the mRNA expression of HSp70, MT10, SOD1, CAT, CYP4Y1, 18S, 28S and EF1 respectively (p-value < 0.05).

Study Areas	B	A	North B	South B	C.M.
B		0.38938	0.80912	0.82204	0.18621
		0.98429	0.08477	0.62401	0.83491
		0.81702	0.76507	0.34974	1.00000
		0.75250	0.58485	0.83746	0.99998
		0.93180	0.99461	0.07364	0.70374
		0.10197	0.03007*	0.58628	0.98983
		1.0E−08*	1.1E−08*	9.0E−09*	2.0E−09*
		0.00316*	0.04827*	0.00049*	0.00025*
A	0.16794		0.03496*	0.03753*	0.00093*
	1.00000		0.23702	0.90302	0.98574
	0.38372		0.16096	0.03005*	0.83098
	0.88266		0.99887	0.16770	0.69212
	0.09419		0.99408	0.00873*	0.24327
	0.39401		0.99050	0.85796	0.27382
	0.31207		1.00000	1.00000	0.99866
	0.55431		0.90730	0.98714	0.96192
North B	0.99999	0.13874		1.00000	0.81118
	0.52802	0.55896		0.71483	0.49883
	1.00000	0.33131		0.96353	0.74902
	0.99769	0.75404		0.09124	0.51931
	0.99998	0.07421		0.02673*	0.45329
	0.00110*	0.25234		0.58990	0.10400
	0.49830	0.99769		1.00000	0.99807
	0.07758	0.78515		0.64439	0.53254
South B	1.00000	0.18752	0.99990		0.79792
	0.31505	0.34065	0.99577		0.99613
	0.99780	0.57931	0.99670		0.33415
	0.96923	0.52290	0.99886		0.88208
	0.99985	0.04895*	0.99891		0.65286
	0.00019*	0.05968	0.95094		0.85920
	0.16494	0.99691	0.96270		0.99882
	0.18419	0.94971	0.99302		0.99980
C.M.	0.97385	0.46502	0.95638	0.98165	
	0.67459	0.70431	0.99941	0.97562	
	0.98642	0.67315	0.98033	0.99973	
	0.99942	0.95491	0.98330	0.90947	
	0.99989	0.08639	1.00000	0.99741	
	0.00193*	0.33325	0.99981	0.89709	
	0.00592*	0.48629	0.30230	0.70497	
	0.01355*	0.36838	0.95443	0.78866	

B: Brucoli; A: Augusta; North B: North Bagnoli; South B: South Bagnoli; C.M.: Capo Miseno.
* The values statistically significant.

ular cells (GC) similar to hemocytes with abundant cytoplasmic granules and peripheral nucleus (Bolognesi and Fenech, 2012). The specimens of gills collected in Brucoli (Fig. 4, Panels A) and in Capo Miseno (Fig. 4, Panels C) showed an overall typical morphology of cells with defined and well-preserved microvilli (MV) (Fig. 4, Panels Aa, Ca) and cilia (CI) (Fig. 4, Panels Aa, Ab, Cb).

Gill ultrastructure of organisms collected from the polluted areas of Augusta, and North and South Bagnoli (Fig. 4, Panels B, D, E) revealed the loss and/or the misalignment of frontal and lateral cilia and disorganization of the microvillus layer with consequent impairment of the absorption surface of the branchial apparatus. Other alterations pertain to the cell nuclei, which lost their ovoid shape while chromatin showed a patchy distribution (Fig. 4, Panels Ba, Ea). Furthermore, the presence of multi-lamellar bodies, RER fragmentation, collapsed Golgi apparatus, dense granules, and cytoplasm vacuolization (Fig. 4, Panels Ba, Bb, Da, Ea) were also found. Infiltration of granular eosinophilic hemocytes (EG) was observed in mussel gills from Augusta and Capo Miseno (Fig. 4, Panels Bc, Cc), whereas basophilic granulocytes (BG) were noted in specimens from Brucoli, and North and South Bagnoli (Fig. 4, Panels Ac, Dc, Ec). Interestingly, several mucus granules were observed inside the gills of Brucoli (Fig. 4, Panel Ab), Augusta (Fig. 4, Panel Bb), and Capo Miseno (Fig. 4, Panel Cb) specimens.

Ultrastructure of *Mytilus galloprovincialis* digestive gland (Fig. 5, Panels A-E) consists of numerous blind-ending tubules (Dimitriadis et al., 2004) surrounded by a collagen sheath (CS) and smooth-muscle fibres (Owen, 1970). The epithelium is composed of digestive cells (DC) and two different types of basophilic cells, termed “basophilic cells” (BC) and “immature basophilic cells” (IBC) (Dimitriadis et al., 2004). The BC possess structural features similar to those of acinar cells of the mammalian exocrine pancreas: pyramidal shape, presence of microvilli in the apical region, cytoplasm filled with flattened cisternae of granular endoplasmic reticulum (ger), extensive Golgi apparatus, secretory vesicles, and lipid droplets (LD) (Fig. 5, Panels Aa, Ba, Ca, Ea, Eb). The IBC are elongated and flagellated cells with a cytoplasm rich in small vesicles and lipid droplets (Fig. 5, Panels Aa, Ba, Ca, Ea, Eb). DC are columnar cells projected into the lumen (LU) of the tubules that present microvilli on the apical surface, Golgi apparatus, smooth endoplasmic reticulum, and free ribosomes scattered in the cytoplasm (Fig. 5, Panels Bb, Cb). The most characteristic features of the DC are the presence of numerous vesicles of different types. The p1 are characterized by irregular shape and electron-dense granular material, the p2 are present in the mid-region of the cell and with a moderate electron-dense content, and the p3 are electron-transparent, spherical organelles (Fig. 5, Panel Aa, Ab, Bb, Ca, Cb, Ea, Eb) (Owen, 1970). The general structural organization of the digestive gland was maintained in mussels from Brucoli (Fig. 5, Panel Aa) and Capo Miseno (Fig. 5, Panels Ca, Cb). However, general disorganization of the tissue characterized by the loss of consistency

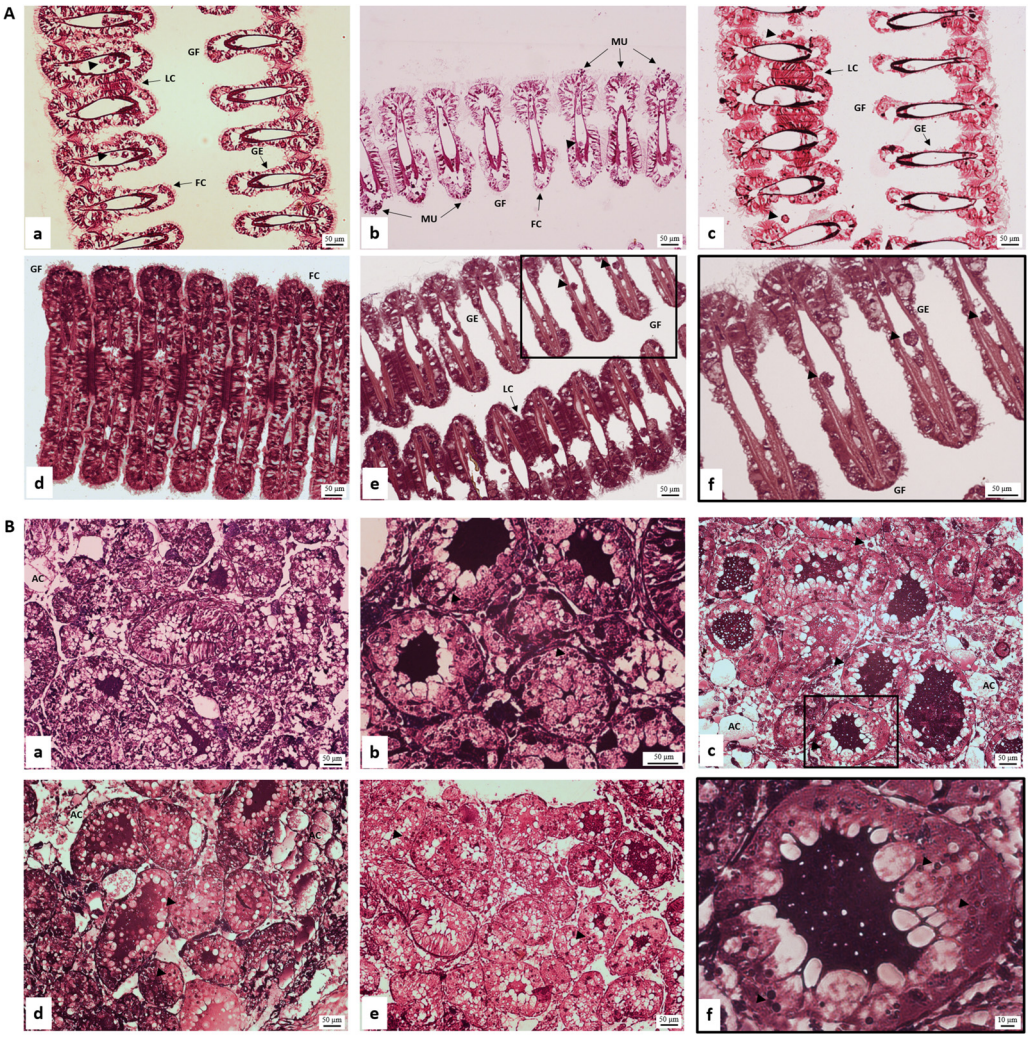

Fig. 3. Representative pictures of gills (A) and digestive gland (B) of *Mytilus galloprovincialis* stained with crystal violet-basic fuchsine. In gills, marked tissue alterations such as impairment of the ciliated epithelium (GE), thinning of filaments (GF), hemocytic infiltration (arrowhead), and hyperplasia (Ad) were observed in particular in those specimens sampled in polluted areas (Ab-f). A general disorganization of the tissue is also present in the digestive gland (Panels Ba-f), with massive infiltration of adipocytes (AC) (Panels Ba, Bc, Bd) and permeation of hemocytes (arrowhead) (see the details in Bf) in the samples of polluted areas. GF, gill filament; FC, frontal cilia; LC, lateral cilia; GE, gill epithelium; MU, mucus granules; AC, adipose cell; black arrowhead, hemocytes infiltration. Brucoli, considered as control area (Aa, Ba), Augusta (Ab, Bb), Capo Miseno (Ac, Bc and its enlargement Bf), North Bagnoli (Ad, Bd), South Bagnoli (Ae and its enlargement Af, Be).

(Fig. 5, Panels Da, Db) and cell vacuolization (Fig. 5, Panels Bb, Cb) was evident in organisms from Augusta and North Bagnoli. Granulocyte infiltrations were observed in all the studied samples (Fig. 5, Panels Ac, Bc, Cc, Dc, Ec).

Altogether, ultrastructural analysis confirmed the observation of optical microscopy and indicated that the gill structure was more compromised than that of the digestive gland, which was strongly modified only in the samples from speciments placed in South Bagnoli.

4. Discussion

The basic goal of eco-toxicology is to predict the effects of pollution and to intervene with the most efficient and effective actions to prevent or remediate any detrimental effect. There are different techniques and tools to assess the impact of pollution on ecosystems, although eco-toxicological approaches are mainly based on the use of biomonitoring and biomarkers (Hamza-Chaffai, 2014).

In this project, we investigated the effects of the presence of contaminated sediments rich in heavy metals such as Fe, Co, Cd, Cu, Zn, and Pb and C10-C50 hydrocarbons on *Mytilus galloprovincialis* mRNA expression and morphology.

Mytilus galloprovincialis is a mussel well recognized as a bio-indicator organism that can be easily caged in specific areas to assess the impact of anthropogenic activities (Maisano et al., 2016; Hamza-Chaffai, 2014; Gorbi et al., 2007). These organisms tend to accumulate pollutants in their tissues (Odzak et al., 1994), often without showing any apparent detrimental effect, probably because they react to the presence of high xenobiotic concentrations by keeping their valves closed so as to reduce interactions with the external environment and minimize the toxic effects (Hietanen et al., 1988). Conversely, at levels of xenobiotics not immediately perceived, these bivalves behave as an early warning system, predicting the effects of low level pollution (Gornati et al., 2016a; Brooks et al., 2015; Hamza-Chaffai, 2014). However, biomonitoring programs based on measuring contaminants in marine organisms could not give enough information about the toxicological significance of pollutants accumulated; therefore, measurable parameters at different levels of biological organization (molecular, cellular, or physiological) are warranted (Hamza-Chaffai, 2014; D'Agata et al., 2014).

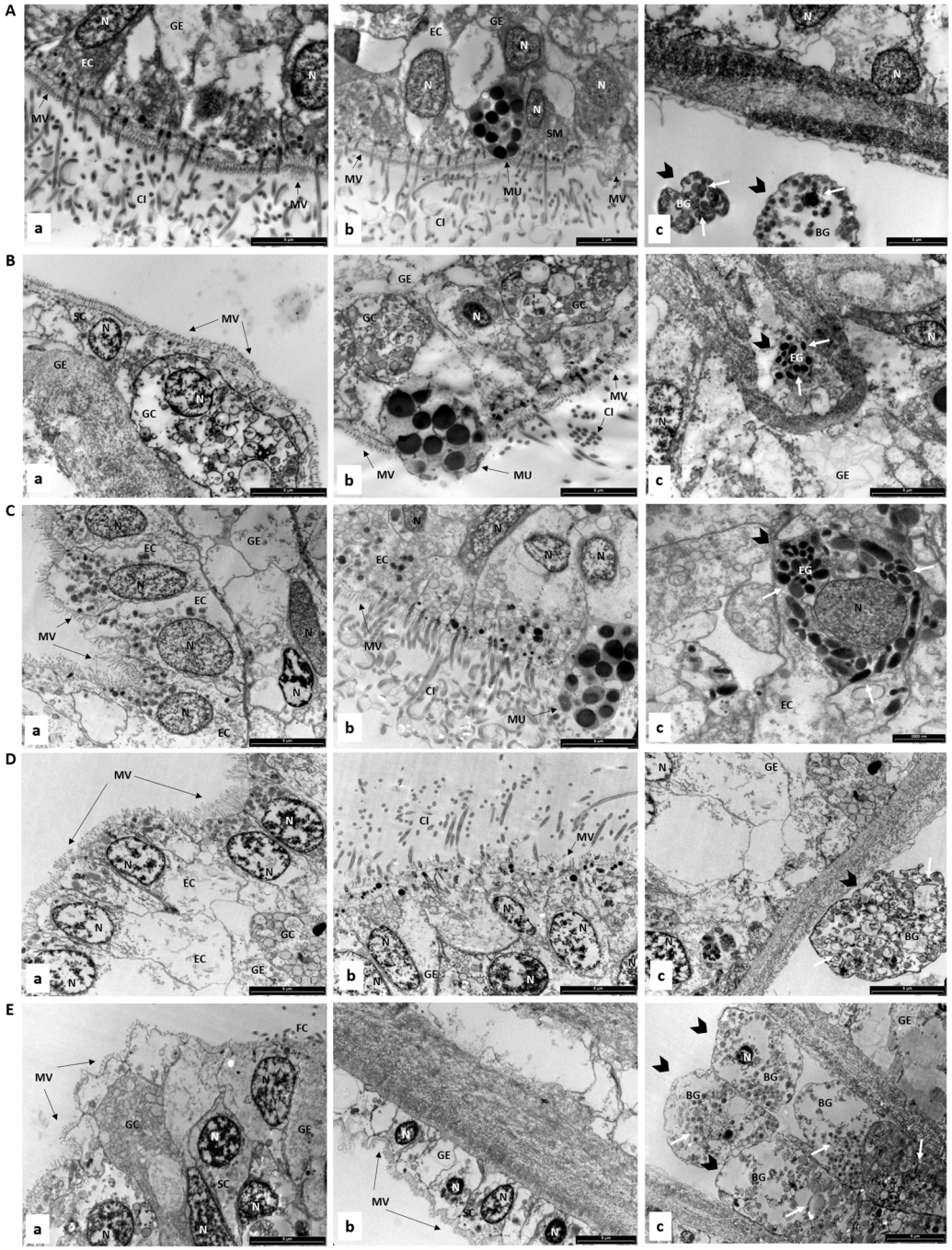

Fig. 4. Examples of TEM micrographs of *Mytilus galloprovincialis* gills. Loss and/or misalignment of frontal (FC) and lateral cilia (CI) and the disorganization of the microvilli (MV) are more evident in those organisms collected from the polluted areas (Bb, Db, Ea) than in the animals sampled in the control area (Aa, Ab). A patchy distribution of chromatin (*) and elevated hemocyte infiltration (arrowhead) are also present. GE, gill epithelium; EC, epithelial cell; GC, granular cell; SC, small cell; N, nucleus; *, condensed chromatin; MV, microvilli; CI, cilia; FC, frontal cilia; MU, mucus granule; BG, basophilic granulocyte; EG, eosinophilic granulocyte; arrowhead, hemocyte infiltration; white arrow, granules. Brucoli (A), Augusta (B), Capo Miseno (C), North Bagnoli (D), South Bagnoli (E).

In this scenario, our study was designed to consider both early transient changes such as gene expression and late and permanent changes such as morphological modifications. The evaluation of mRNA levels is widely used as a biomarker in many research fields: medical diagnosis (Madu and Lu, 2010), animal welfare (Gornati et al., 2004), toxicology (Gornati et al., 2016b; Dondero et al., 2005), and environmental monitoring (Lacroix et al., 2014; Coimbra Rola et al., 2012; Banni et al., 2011, 2007; Quirós et al., 2007). The same literature confirms the ability of mRNA evaluation, when associated to a multivariate analysis, to highlight side-effects. An advantage of mRNA evaluation that makes it so attractive is certainly its quite early response, which can be easily and reliably detected by qPCR. Subsequently, a robust panel of genes was selected and housekeeping and target genes were defined (Kozera and Rapacz, 2013; Hruz et al., 2011; Taylor et al., 2010). These characteristics can provide clear knowledge of the context and, if necessary, immediate intervention is possible to avoid permanent damage (Gornati et al., 2004). On the other hand, to be useful, mRNA expression has to be assessed within a fairly short time, for at least two reasons. The first is related to the adaptive processes developed by the mussels (Gazeau et al., 2010) and the second is due to the highly sophisticated cell mechanism governing the mRNA synthesis, rendering

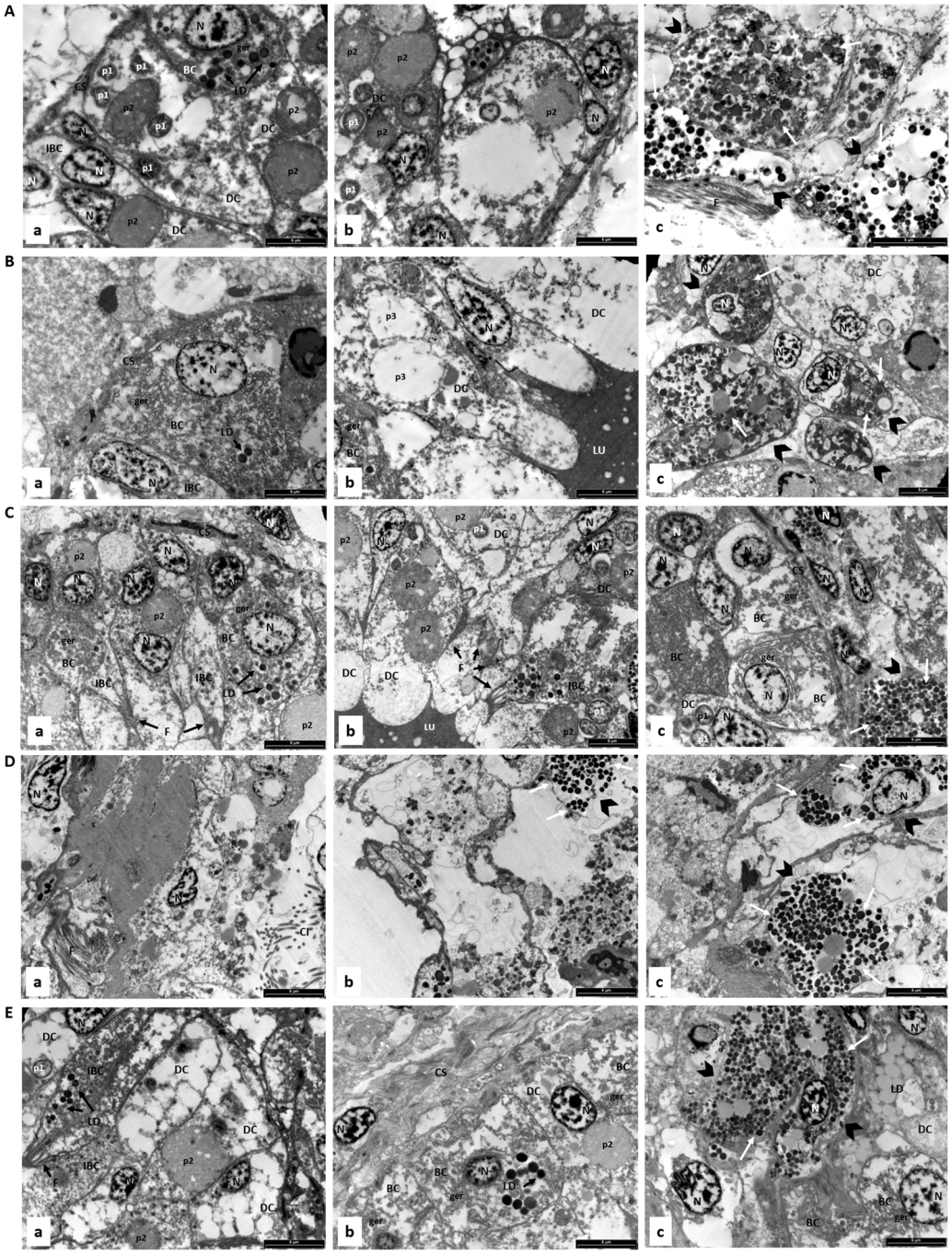

Fig. 5. Examples of TEM micrographs of *Mytilus galloprovincialis* digestive gland. An evident general disorganization of the tissue (D) accompanied by cell vacuolization (Bb, Db) of digestive tubules were observed. Massive hemocyte infiltrations (arrowhead) (Bc, Dc, Ec) were particularly evident in the animals sampled in Augusta (B), South Bagnoli (D), and North Bagnoli (E). DC, digestive cell; BC, basophilic cell; IBC, immature basophilic cell; F, fibres; CS, collagenous sheath; N, nucleus; LD, lipid droplets; ger, granular endoplasmic reticulum; CI, cilia; LU, lumen; p1, vesicles type 1; p2, vesicles type 2; p3, vesicles type 3; arrowhead, hemocyte infiltration; white arrow, granules. Brucoli (A), Augusta (B), Capo Miseno (C), North Bagnoli (D), South Bagnoli (E).

gene expression alone not informative enough (Vogel and Marcotte, 2012). In fact, it is known that the morphology of cells with the same genome may be different depending on their protein expression and cells with different transcriptomes may present a similar phenotype (Kendrick, 2014; Schwanhäusser et al., 2011; Lundberg et al., 2010; Tian et al., 2004). In this study, we combined two different methods, transcriptomic and morphological analysis, with the purpose to determine whether cell morphology and the structural organization of our animal model are related to gene expression in outdoor experiment. Consequently, our aim was to understand whether quantitative evaluation of mRNA is a useful endpoint for providing valuable feedback about the eco-toxicological effects in outdoor experiments.

Capo Miseno, an area not affected by petrochemical contamination, was initially proposed as a control site for North and South Bagnoli. Unfortunately, sediment analysis showed a high amount of heavy metals (Cd, Pb, Zn). These data forced us to consider Brucoli as a reference site and Capo Miseno as a study area together with Augusta, North and South Bagnoli. In fact, concerning mRNA expression both for the mussels caged in Capo Miseno and in the other areas, a marked discrepancy was found between gills and digestive gland, in particular for those genes involved in protein

machinery (*18S, 28S* and *EF1*). It is known that *18S, 28S*, and *EF1* mRNA expressions are generally related to cell proliferation and could be the consequence of tissue regeneration (Talukder et al., 2001; Nikolov and Dabeva, 1983; Bowman and Emerson, 1977; Schneider and Shorr, 1975).

In accordance with metal concentrations found in the polluted areas, an upregulation, though not statistically significant, was found for *MT10* both in gills and in digestive gland, while *HSP70, SOD1, CAT*, and *CYP4Y1* showed occasional variances not related to a particular area and/or particular contamination. These results are in accordance with data reported in the literature for field experiments (Maisano et al., 2016; Lacroix et al., 2014; Coimbra Rola et al., 2012) and highlight the importance of mRNA expressions as biomarkers in environmental monitoring programs. Modification in mRNA expression, even if transient and barely detectable, can anticipate changes in histomorphological architecture; therefore, in addition to molecular analysis, we also documented macro- and microscopic changes. Sketchy inspection revealed that perturbations in the general health status of mussels occurred after 60 days of exposure in the polluted areas (Augusta, North and South Bagnoli). Optical microscopy analysis showed relevant tissue modifications in gills and digestive gland of the mussels caged in the polluted sites, confirming the macroscopic observations, even though mussels caged in Capo Miseno sometimes showed evidence of a consistent infiltration of hemocytes and impairment of the ciliated epithelium (Indumathi et al., 2011). TEM investigation, in addition to corroborating the observations obtained by light microscopy, showed a consistent infiltration of granular hemocytes, eosinophil cells, in particular, suggesting that the immune system was activated as a general response to stressful stimuli such as the presence of toxic compounds (Gornati et al., 2016a; Renwrantz et al., 2013; Pruzzo et al., 2005). The presence of several mucus granules seems to be associated with heavy metal pollution (Hietanen et al., 1988). Alterations involving gill epithelial cells and digestive gland parenchyma, consist of multi-lamellar bodies, RER fragmentation, collapsed Golgi apparatus, dense granules, and cytoplasm vacuolization and are indicative of an advanced apoptotic state.

Taken together, these findings support the evidence of an initial inflammatory response by the cells that can lead to apoptosis for the samples caged in all the polluted areas.

5. Conclusions

This research was part of a national project funded by the Italian Ministry on Systems Biology. The project aims to understand how marine pollution modifies the biological processes in aquatic species and to evaluate the potential effectiveness of different biotechnological strategies in controlling and reducing the impact of marine pollution on the biological compartment.

In this work, we present results demonstrating that the exposure of *Mytilus galloprovincialis* to petrochemical pollutants induced changes on the expression of genes involved in protein machinery. The outcome of these changes are the morphological alterations found both in gills and in digestive gland.

Overall, the application of a multi-disciplinary approach proved to be effective for assessing the biological effects of contaminations on the health of aquatic organisms and thus suitable to be applied in eco-toxicological studies. Although affected by several uncontrolled environmental variables, such as marine environment parameters (salinity, temperature), sea storms, and currents, inability to place and/or retrieve the cages on time, seasonality, and even theft, the assessment of mRNA can represent a useful endpoint for an integrated estimation of the overall threats to the sea environment within a field research approach. In the near future, a series of controlled mesocosm-scale experiments using the same animal model to measure the effects of sediments collected from the contaminated areas will be set up to corroborate the results from the present research.

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