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Biomarkers of Environmental Marine Pollution for Nutrient, Hazard Analysis and Critical Control Point Process along Calabrian Coasts

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Introduction

Non-communicable diseases (NCDs) are the leading cause of death worldwide, causing a number of deaths that exceeds the sum of all other diseases and a more significant impact of these forms of disease falls on low-and middle-income populations. NCDs have reached epidemic proportions, but they could easily be avoided and significantly reduced by the implementation of preventive policies to eliminate or reduce risk factors.

Presently, we do not only need safe food but food that can help the consumer maintain a good state of health and prevent NCDs, the identification of new biomarkers is required to apply the NACCP process, for total quality management (TMQ), and high nutritional levels.[1] (Figure 1).

The present era is called Anthropocene, due to the impact of human activities on the entire terrestrial ecosystem. The territorial, structural and climate changes operated by humans have consequences on all other living organisms.[2] When a toxic compound penetrates an ecosystem, it can cause a number of alterations at different levels of structural complexity, ranging from molecular damage to modifications at the level of organisms, populations or communities. Some of these living organisms can provide information about the environment in which they live, therefore, they are referred to as sentinel organisms.[3] In these organisms, some parameters are well detectable as biological markers or indicators, providing information on the effects of the contaminants on biological systems.

The biomarkers or stress indexes can be defined as alterations, induced by a contaminant, at the level of molecular or cellular components, of a structure or function, which can be detected and quantified in a sentinel organism. Simultaneously to the negative impact of the pollutant, the organism develops adaptive responses to stress that aim to bring to a state of homeostasis.

The term biomarker has been defined by the National Academy of Sciences in the USA as follows: "A biomarker is a xenobiotically induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample".[4]

Such responses tend to decrease the toxic effect of the pollutant through the involvement of multi-enzymatic systems such as metallothioneins,[5] acetylcholinesterase[6] and cytochromes.[7] These enzymatic systems can detoxify the organism entirely or in part.

Aquatic organisms are subjected to a different kind of stress due to human activities. It's difficult to evaluate the impact of anthropogenic pollution on the communities belonging to aquatic ecosystem,[8,9] and investigations involving chemical analysis only, are not sufficient to study problems of this magnitude, since pollutants can have deleterious effects on living organisms even when present in low concentrations.[10] Considering these limits, recent environmental monitoring programs utilize "biomarkers"[11] as a methodological approach, in order to evaluate the organism's response to environmental stress of chemical or physical nature.[12]

Because of their filtering habits, bivalve molluscs represent a useful sentinel species for assessing marine contamination.[13,14,15,116]

Mytilus galloprovincialis is a specie widely used as a bio-indicator organism in environmental monitoring, due to the numerous advantages it offers,[17] not least its high performance in histological studies and biochemical analysis.[18]

Digestive gland of this cosmopolitan bivalve species has already been largely used in several previous marine studies. In fact, in the digestive gland the mussels accumulate high levels of heavy metals, because this organ represents their most important detoxifying system.[19,20]

Metallothioneins are inducible proteins and represent a suitable potential biomarker of metal environmental pollution.[21,22] It is widely known that these proteins have biological functions related to homeostatic metals control[23] and detoxification of excess metals.[24,25,26]

Molecular biology recent advances allowed a precise characterization of different protein isoforms of metallothioneins[27,28,29,30]

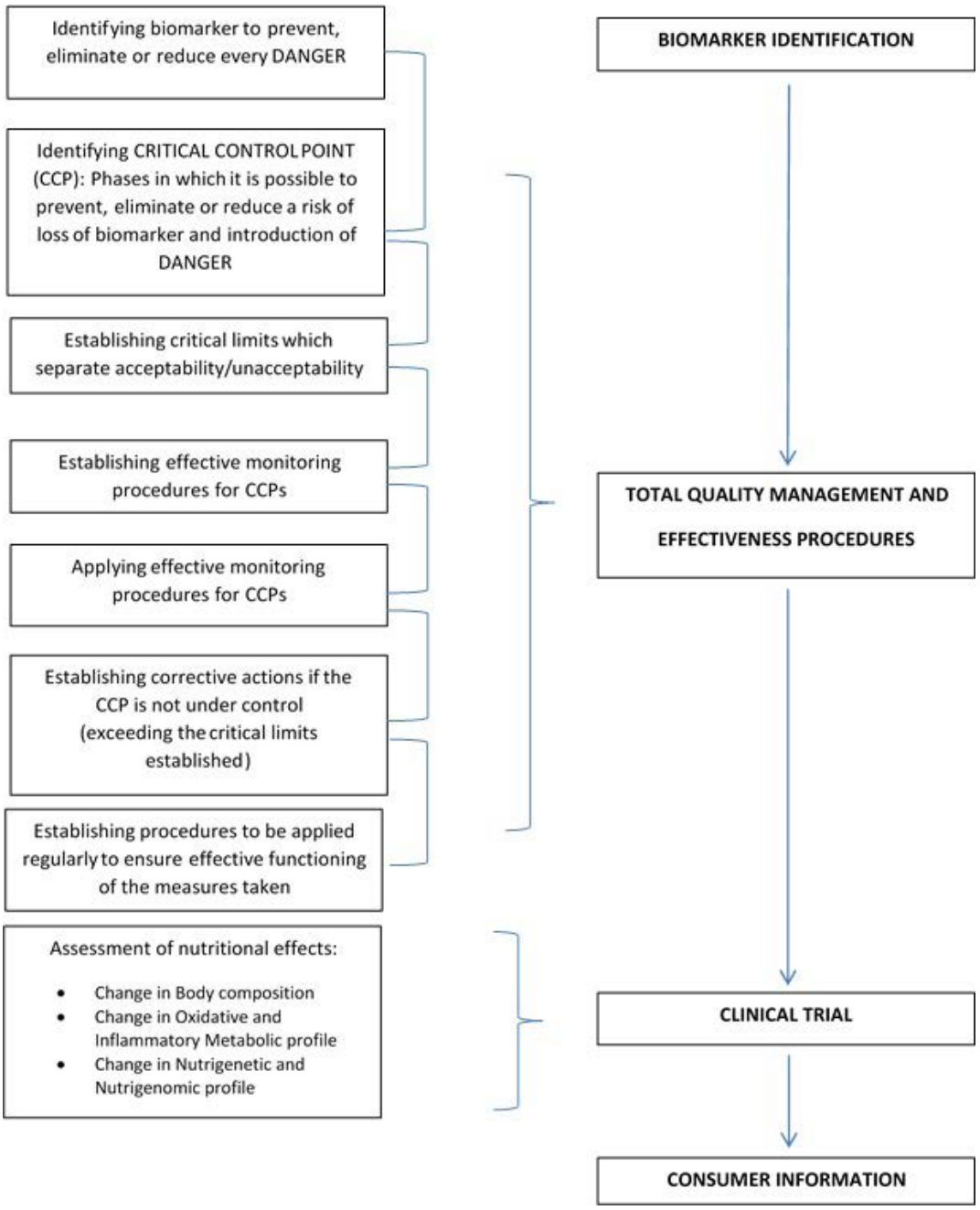

Figure 1. The NACCP process

and clarified their role in homeostatic and detoxification mechanisms[31,32] also identifying the expression of specific genes associated with cellular functions.[33,34,35]

Different gene isoforms in *Mytilus galloprovincialis* have been identified.[36] It has been documented in literature that essential metals exposure, such as zinc (Zn), induces a rapid gene MT10 response,[37] while MT20 gene isoform is specifically induced by non-essential metals, such as copper (Cu), and cadmium (Cd).[38]

AChE enzyme is responsible for hydrolyzing the neurotransmitter acetylcholine into choline and acetic acid.[39] AChE is usually located in the membranes (e.g. of erythrocytes) of vertebrates and non-vertebrates; the enzyme controls ionic currents in excitable membranes and plays an essential role in nerve conduction processes at the neuromuscular junction. The inhibition of AChE is linked directly with the mechanism of toxic action of organophosphate and organochlorinated insecticides, by irreversible or reversible binding to the catalytic site of the enzyme and potentiation of cholinergic effects.[40] Marine bivalves such as clams, oysters and mussels are widely used as bioindicators of contamination in the monitoring of pollutant effects.[41,42,43] However, only a few studies have examined the effects of organophosphate insecticides in aquatic invertebrates[44,45,46] and particularly in mussels, which are widely used in pollution monitoring programs.[47] Apart from the insecticides, a few other contaminants, including cadmium, mercury, lead and copper were found to show anticholinesterase activity.[48]

It's well known that the CYP1A subfamily plays a key role in the biotransformation of contaminants like dioxins, furans, polychlorinated biphenyls and polycyclic aromatic hydrocarbons.[49] The induction of CYP1A is triggered via the cytosolic aryl hydrocarbon (Ah) receptor which is activated by exposure of organisms to such pollutants.[50] The measurement of the induction of CYP1A in terms of 7-ethoxyresorufin O-deethylase (EROD) activities is utilized as a potential biomarker for marine pollution monitoring.[51] Extensive studies were carried out to investigate changes on EROD in the freshwater bivalves, exposed to different pollutants (Arochlor 1260, CB-153 and CB-126, pp¢ DDT, Chlorpyrifos, Carbaryl) at laboratory conditions.[52]

The aim of this work is to provide further data for the evaluation of the ecotoxicological status of the Calabrian coastal waters

using assays of metallothionein gene expressions, acetylcholinesterase and cytochrome P450 enzyme activities, using, as a target organ, the digestive gland of a population of transplanted mussels.

In this work, we describe the results obtained using a range of biomarkers in transplanted mussels in pilot monitoring stations already identified by the National Coastal Waters Monitoring Programme (Law 979/82). For this study, seven Calabrian seaside locations have been identified: four situated on the Ionic coast (Caulonia, Crotone, Isola Capo Rizzuto, and Pellaro) and three on the Tyrrhenian coast (Cetraro, river Mesima's mouth, and Vibo Marina) (Figure 2, Table 1).

We have considered critical areas Crotone and river Mesima's mouth; and white areas Cetraro and Isola Capo Rizzuto.

Crotone was chosen because it was an important industrial site (Pertusola – Montedison), river Mesima's mouth was chosen because it has been an intensive agricultural area.

Cetraro and Isola Capo Rizzuto were chosen for their statement as protected marine areas.

The selection of the surveyed areas (sampling areas) was carried out on the basis of the knowledge of the different territorial realities and of the results obtained from the previous monitoring programs. The aim is to identify areas subject to specific stresses,

Table 1. Geographic coordinates of study areas .

Study areas	latitude	longitude
Caulonia	38.3817100°	16.4095100°
Cetraro	39.5166000°	15.9415800°
Crotone	39.0807700°	17.1276400°
Isola Capo Rizzuto	38.9584400°	17.0924200°
Pellaro	38.0100000°	15.3800000°
River Mesima's mouth	38.3000000°	15.5500000°
Vibo Valentia	38.6761800°	16.1009400°

critical areas, and areas that are subjected to inferior anthropic impacts, thus assuming the function of control areas or *white areas*. The latter have been identified mainly within Marine Protected Areas (AMPs).[53]

Materials and Methods

Study areas and samples

In the summer, mussels belonging to the species *Mytilus galloprovincialis* were acquired from a mussel farming facility with a

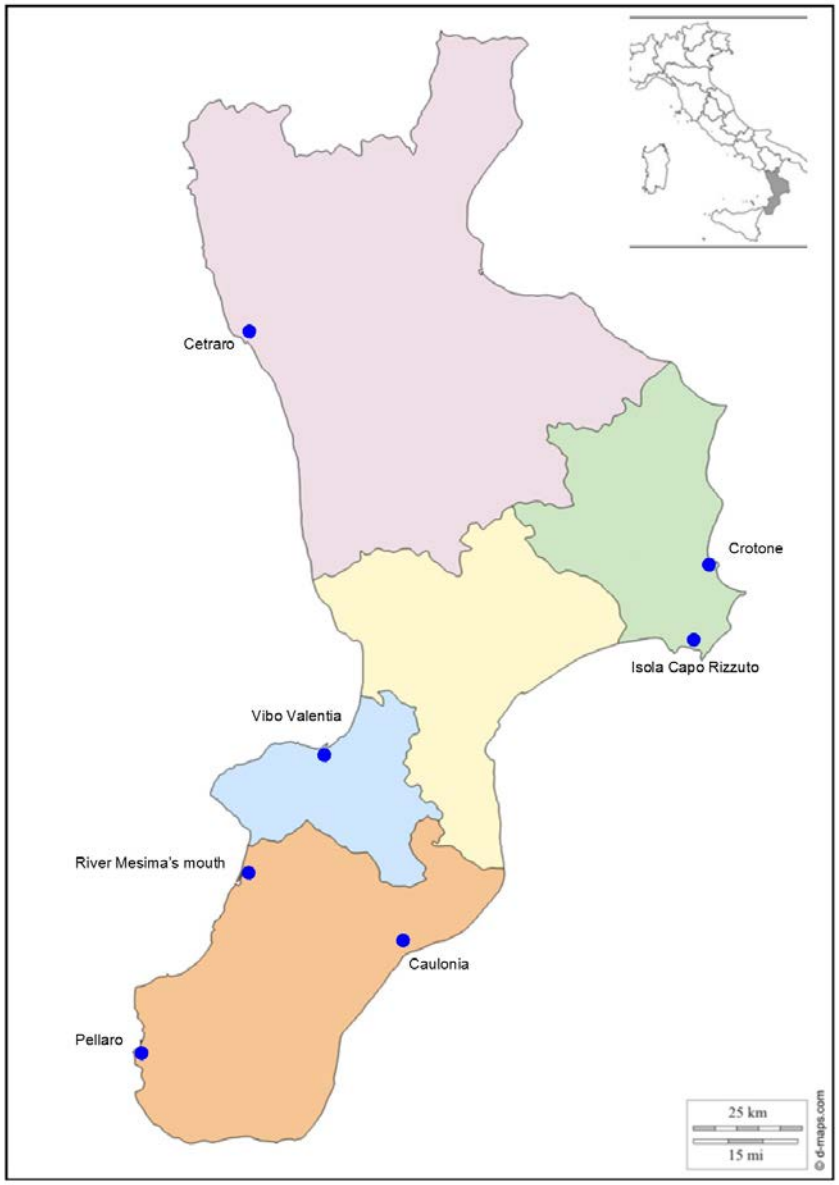

Figure 2. Study areas along the coastline of Calabria, Italy

long line system, situated in the coastal waters close to Crotone. Seven aliquots (about 10 Kg) were positioned, one at each of the seven study areas [see table 1], with a further aliquot used as a control sample (non-transplanted mussels). All the transplanted mussels were left to stabilize for twelve weeks according to the *Mussel Watch* protocol;[54] the control, instead, were immediately processed.

From a pool of 30 individuals for each monitoring station and control aliquot, the digestive glands were separated from other soft tissues and, for each aliquot, chemical and molecular analyses were carried out. The biomarkers analyzed, assayed according to standardized methods reported in the literature, were respectively: metallothionein (MT10 and MT20) gene expressions,[55] and acetylcholinesterase[56] and cytochrome P450 activities.[57]

RNA extraction, cDNA synthesis, quantitative real time PCR analysis of MT genes

Total RNA has been obtained from 30 mg of pooled digestive glands by RNA aqueous-Micro Kit (Ambion); RNA clearness and concentrations have been established by absorbance (A_{260}/A_{280}) measurement. cDNA has been synthesized from 0,5-1 µg of RNA by High Capacity RNA to cDNA Kit (Applied Biosystems), and gene expression of MT10 (GenBank n° AY 566248) and MT20 (GenBank n° AY 566247) mRNA is quantitatively measured by termocycler real-time PCR (Biorad, mod.IQ5), with SYBR® Green PCR Master Mix (Applied Biosystems) and primers: MT10: FW:5'-GGGCGCCGACTGTAAATGTTC-3'; MT10: RW:5'-CACGTTGAAGGYCCTGTACACC-3'; MT20: FW:5'-TGTGAAAGTGGCTGCGGA-3'; MT20: RW:5'-GTACAGCCACATCCACACGC-3'.

Gene targets MT10 and MT20 mRNA expression levels are normalized with a 18S rRNA fragment (GenBank n° L33452) as housekeeping in presence of primers: 18S FW: 5'-TCGATGGTACGTGATATGCC-3'; 18S RW: 5'-CGTTTCTCATGCTCCCTCTC-3'.

Enzymatic activity of acetylcholinesterase

Using the colorimetric method first developed by Ellman *et al*. (1961),[58] the activity of AChE can be measured in digestive glands of mussels. A synthetic substrate for AChE, acetylthiocholine iodide (AtCh), is broken down to thiocholine and acetate. When let thiocholine reacts with dithiobisnitrobenzoate (DTNB), 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate are produced. The latter has a yellow color that is detectable by a spectrophotometer at 405 nm. The intensity of the yellow color reflects the activity of AChE. The method has been opportunely modified to microplate.[59] Prior to AChE biochemical analysis, the tissue was ground in (1:1) Tris-HCL 10 mM pH 7.6, KCL 0.15M, Sucrose 0.5 M, Aprotinin 16.8 mU buffer. Each sample obtained by 1 g of tissue homogenization in 50 mM TRIS pH 7.5, 1 mM EDTA, and 150 mM NaCl buffer, incubated on ice for 20 min, has been centrifuged at 9.000 g for 30 min at 4°C. The supernatant was removed and used to determine AChE activity. Protein content has been spectrophotometrically (562 nm) determined by BCA assay as previously described.[60]

Briefly: 50 µl of three different concentration of each sample for well reacted with 200 µl of the assay buffer: AtCh 0.5 mM, DTNB 0.3 mM, and phosphate buffered saline (PBS) 0.1 M pH 7.2. Reading assay has been performed for 30 min. The ΔOD/min has been calculated from the linear portion of the curve after the substrate autohydrolysis subtraction. Results are expressed as triplicate mean values +/- mean standard error (S.E.M.) normalized for *Torpedo Californica* AChE content and expressed as µmoles (micromoles) of substrate produced for min for mg of protein.

Enzymatic activity of cytochrome P450

Enzymatic activity of the CYP450, CYP1A1 isoform has been determined by a fluorimetric Kit (IZKUS Environment). EROD[61] activity has been determined on 50 µl of supernatant (obtained by 1 g of tissue homogenization in 50 mM TRIS pH 7.5, 1 mM EDTA, and 150 nM NaCl buffer, and centrifuged at 9000 g for 20 min., 4°C) and 10 µM NADPH in 100 mM of KH_2PO_4, with 7-ethoxyresorufin, to start the reaction. The assay was carried out for 40 minutes by a fluorimeter reader (Jasco, FP-920). The ΔOD/min has been calculated from the linear portion of the curve. EROD activity has been determined in tissue extracts. Results are expressed as triplicate mean values ± mean standard error (S.E.M.) and expressed as picomoles (pmoles) of substrate produced for min for mg of protein. This method agrees with ISO standard for PAH and PCBH water pollution determination.

Chemical analysis

Heavy metals analysis was carried out on lyophilised tissue. Samples were digested with HNO_3 using a microwave (Ethos Touch Control, Milestone) oven with electronic temperature and pressure controls, in order to avoid loss of the analytes during the heating process. The concentration of individual substance was determined using optical ICP[62] (mod. Optima 2100 DV, Perkin Elmer). The compounds of organic nature (carbamates, organic phosphates, halogenated hydrocarbons) were determined using gas chromatography linked to mass spectroscopy.[63]

Statistical analysis

The statistical analysis of data was done using one-way analysis of variance (ANOVA) followed by Student's T *post-hoc* test. Correlation coefficients (R) were determined using regression analysis at a significant level of 95% confidence intervals on mean values. Means were referred to three biological repeats for each set of three independent experiments ± mean standard error (S.E.M.). The significance of results was ascertained at P value: $p<0.05$.

Results

Levels of expression of the MT10 and MT20 genes

Data from gene expression of MT10 and MT20 mRNAs, obtained by experimentation with Q-PCR, was normalized against 18S ribosomal RNA and reported as Normalized Fold Expression, compared to the control, which is reported as a value of 1.

Figure 3 shows the level of MT10 mRNA expression. The highest levels of MT10 mRNA expression were found, in the order, in Crotone and Cetraro monitoring stations. Levels of gene expression slightly above the control were found in the monitoring stations of river Mesima's mouth and Caulonia. Lower levels than to control were found in Isola Capo Rizzuto, Pellaro, and Vibo Marina areas.

On the basis of the statistical test used to analyze the experimental data, the value observed in river Mesima's mouth and Caulonia stations was not considered statistically significant.

Figure 4 shows the level of MT20 mRNA expression. Mussels positioned at the Crotone monitoring station showed the highest level of expression, followed by Isola Capo Rizzuto. At Vibo Marina and Cetraro were found levels of expression slightly above the control, while in the remaining three study areas: Caulonia, Pellaro, and river Mesima's mouth were measured the lowest expression's levels of this metallothionein.

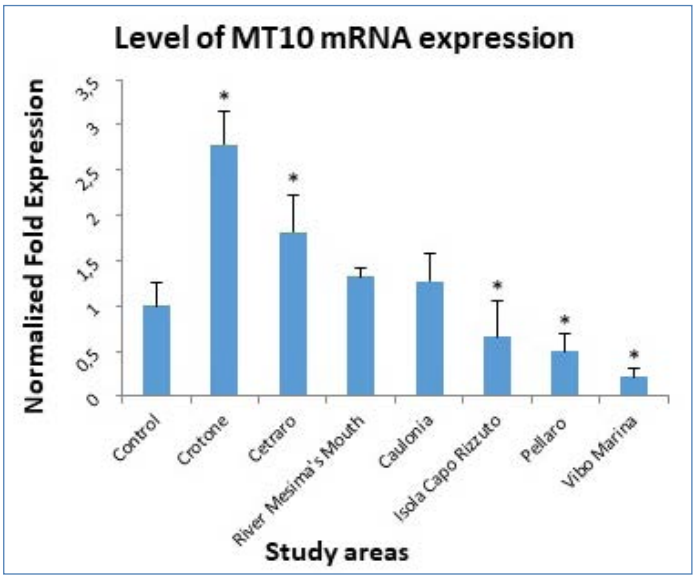

Figure 3. Levels of Metallothionein (MT10) mRNA determined in the digestive gland of mussels. Results are reported as average values of at least three different determinations with the indication of the standard error mean (SEM) and expressed as normalized fold expression compared to the control. The significance of the data was evaluated by p-value (*p<0.05).

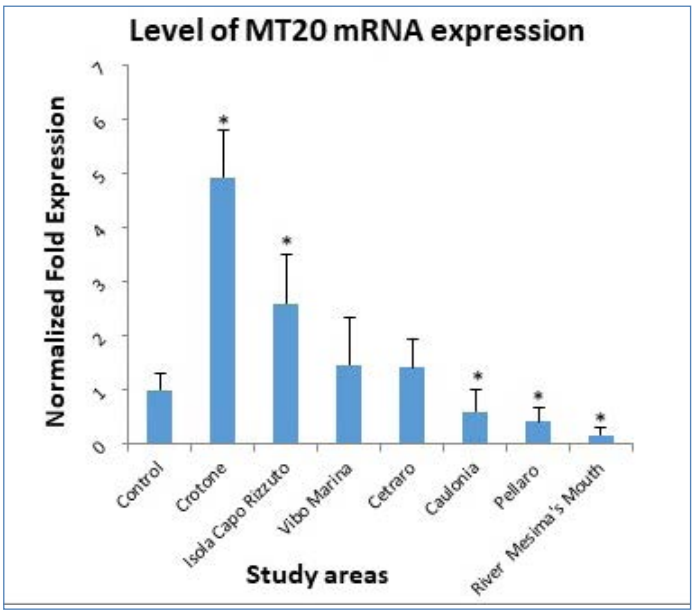

Figure 4. Levels of Metallothionein (MT20) mRNA determined in the digestive gland of mussels. Results are reported as average values of at least three different determinations with the indication of the standard error mean (SEM) and expressed as normalized fold expression compared to the control. The significance of the data was evaluated by p-value (*p<0.05).

On the basis of the statistical test used to analyze the experimental data, the value observed in Cetraro and Vibo Marina stations was not considered statistically significant.

Acetylcholinesterase enzyme activity

Figure 5 shows the data for acetylcholinesterase enzyme activity. Values obtained are lower than the control sample in all study areas, exceeding the highest threshold (20% inhibition) used by the U.S. EPA (1998)[64] for the definition of "biologically significant inhibition" for acetylcholinesterase activity.

At each station, the AChE activity was less than control but, by the statistical test used to analyze the experimental data, the value observed in Vibo Marina station was not considered statistically significant.

Cytochrome P450 enzyme activity

Figure 6 shows the results of the determination of enzyme activity of the cytochrome P450 isoform CYP1A1. Results show a marked increase in CYP1A1 activity in all sites.

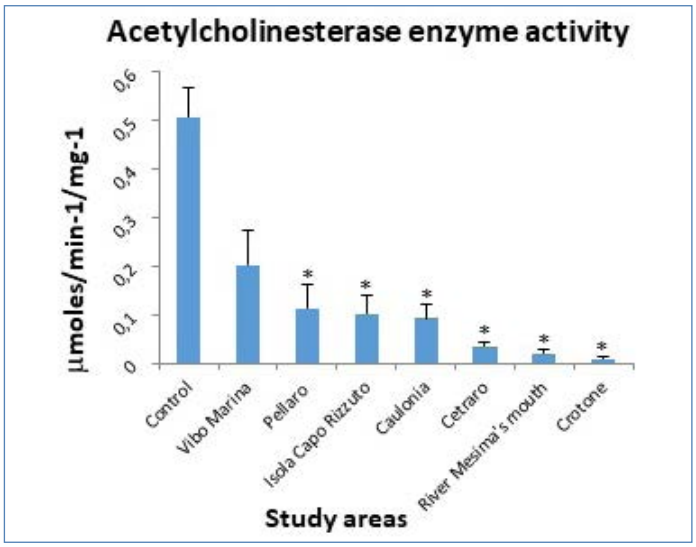

Figure 5. Acetylcholinesterase (AChE) enzyme activity determined in the digestive gland of mussels. Results are reported as average values of at least three different determinations with the indication of the standard error mean (SEM) and expressed as µmoles/min^{-1}/mg^{-1}of protein normalized for *Torpedo Californica* AChE content. The significance of the data was evaluated by *p*-value (* p<0.05).

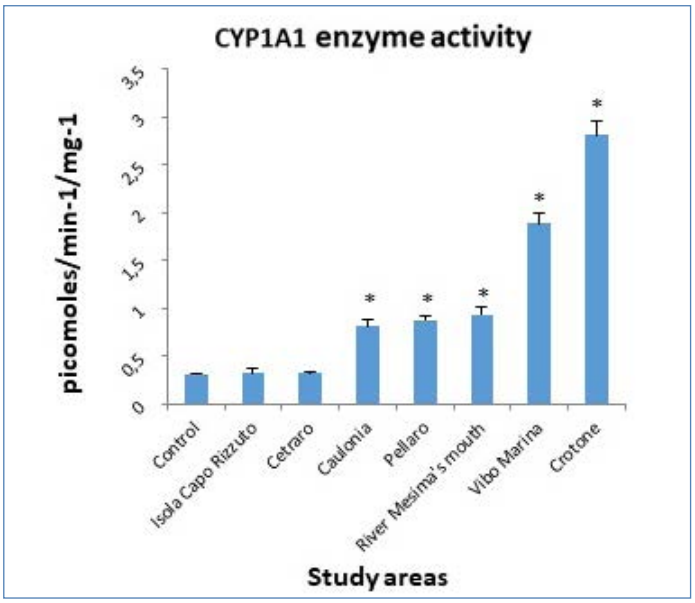

Figure 6. Cytochrome P450 (CYP1A1) enzyme activity determined in the digestive gland of mussels. Results are reported as average values of at least three different determinations with the indication of the standard error mean (SEM) and expressed as pmoles/min-1/mg-1of protein. The significance of the data was evaluated by p-value (* p<0.05).

Table 2. Average of concentrations (µg/g wet weight) of metals from the digestive gland of mussels. The significance of the data was evaluated by p-value (* p<0.05).

Study area	Ag	Al	As	Cd	Cr	Cu	Fe	Hg	Ni	Pb	Va	Zn
Control	0.11	19.5	8.3	0.85	0.46	3.63	370.2	0.03	2.6	0.8	0.43	90.2
Caulonia	0.031	311.21	3.83	0.5	0.73	4.7	423.65*	0.11	0.54	0.82	0.44	92.29
Cetraro	0.022	200.83	13.56	0.71	1.68	3.41	243.65	0.21	1.48	1.02	1.12	122.12*
Crotone	0.024	290.5*	8.4	0.84	0.55	4.36*	60,.2	0.06	2.11	1.05*	1.01	145.02*
Isola Capo Rizzuto	0.012	98.15*	17.35*	0.51	7.72*	4.7*	106.52	0.05	1.93	0.52	0.86*	89.42
Pellaro	0.082	231.4	9.8*	0.8	0.85*	3.2	212.47	0.1*	0.81	0.3	1.01*	75.26
River Mesima's mouth	0.12	178.33	9.2	0.9	0.66	3.63	333.55	0.2	2.31	0.8	0.13	92.3
Vibo Marina	0.12	110.73	5.2	0.72	0.44	2.15	107.59	0.15	0.69	0.22	0.19	54.3

However, for that concern the values observed in Cetraro and Isola Capo Rizzuto areas, by the statistical test used to analyze the experimental data, these were not considered statistically significant.

Microelements analysis

Table 2 shows the concentration (expressed as µg/g wet weight) of microelements, such as heavy metals and metalloids, determined in the digestive gland of *Mytilus galloprovincialis* in the seven study areas and control sample. In all stations examined, the concentrations of the inorganic chemical elements measured differed more or less markedly from the control sample. Considering the concentration values higher than the control, we note that: Ag concentrations were found to be slightly higher than control (0.11 µg/g w.w) in Vibo Marina and river Mesima's mouth stations (being 0.12 µg/g w.w in both stations); Al concentrations were found to be considerably higher than control (19.5 µg/g w.w) in all the stations examined (with values ranging from 98.15 µg/g w.w in Isola Capo Rizzuto to 290.5 µg/g w.w in Crotone); As concentrations were found to be slightly higher than control (8.3 µg/g w.w) in Crotone, river Mesima's mouth, and Pellaro stations (8.64, 9.2, and 9.8 µg/g w.w respectively), and notably higher than control in Cetraro and Isola Capo Rizzuto stations (13.56 and 17.35 µg/g w.w respectively); Cd concentration was found to be slightly higher than control (0.85 µg/g w.w) only in river Mesima's mouth station (0.9 µg/g w.w); Cr concentrations were found to be slightly higher than control (0.46 µg/g w.w) in Crotone, river Mesima's mouth, Caulonia, and Pellaro stations (0.55, 0.66, 0.73, and 0.85 µg/g w.w respectively), notably higher than control in Cetraro station (1.68 µg/g w.w), and considerably higher than control in Isola Capo Rizzuto station (7.72 µg/g w.w); Cu concentrations were found to be slightly higher than control (3.63 µg/g w.w) in Crotone (4.36 µg/g w.w), Caulonia, and Isola Capo Rizzuto stations (being 4.7 µg/g w.w in both stations); Fe concentration was found to be higher than control (370.2 µg/g w.w) only in Caulonia station (423.65 µg/g w.w); Hg concentrations were found higher than control (0.03 µg/g w.w) in all the station examined, precisely: slightly higher in Isola Capo Rizzuto and Crotone stations (0.05 and 0.06 µg/g w.w respectively), notably higher in Pellaro, Caulonia, and Vibo Marina stations (0.1, 0.11, and 0.15 µg/g w.w respectively), considerably higher in river Mesima's mouth and Cetraro stations (0.2 and 0.21 µg/g w.w respectively); none of the stations tested were found to have Ni concentrations higher than control; Pb concentrations were found to be slightly higher than control (0.8 µg/g w.w) in Caulonia station (0.82 µg/g w.w), and notably higher than control in Cetraro and Crotone (1.02 and 1.05 µg/g w.w respectively); Va concentrations were found to be slightly higher than control (0.43 µg/g w.w) in Caulonia station (0.44 µg/g w.w), notably higher than control in Isola Capo Rizzuto station (0.86 µg/g w.w), and considerably higher than control in Pellaro, Crotone (1.01 µg/g w.w in both stations), and Cetraro (1.12 µg/g w.w); finally, Zn concentrations were found to be slightly higher than control (90.2 µg/g w.w) in Caulonia and river Mesima's mouth stations (92.29 and 92.3 µg/g w.w respectively), and notably higher than control in Cetraro and Crotone stations (122.12 and 145.02 µg/g w.w respectively).

Organic contaminants

Regarding the analyzed organic compounds, the highest concentration of Chlorpyrifos and Chlorfenvinphos (banned by the European Union since 2003) compounds were detected at the river Mesima's mouth station. Meanwhile the concentration of 4,4'-Dichlorodiphenylethylene (4,4'DDE) was below the threshold value and the concentration of 2,4'-Dichlorodiphenylethane (2,4'DDE) was significantly above this value. In regards to Pellaro, while the concentrations of Chlorpyrifos and Chlorfenvinphos was below the threshold value, the concentrations of 4,4'-DDE and 2,4'-DDE was significantly above this value. Crotone showed the concentrations of Chlorpyrifos and Chlorfenvinphos significantly above the threshold value, and the highest concentration of 4,4'-DDE and 2,4'-DDE. In Caulonia the concentrations of both organophosphate and organochlorinated compounds were significantly higher than control. Instead, in Isola Capo Rizzuto the levels of all four compounds were below the threshold value. Lastly, Cetraro showed the concentrations of organophosphate compounds significantly higher than control, while the concentration of organochlorinated compounds were below the threshold value. (Table 3).

Discussion

The results obtained from the assays carried out in the present study confirmed the validity of proteins (MT10 and MT20), and enzymes (AChE and CYP1A1) taken into account as pollutant biomarkers.

As described in the literature, the MT10 gene expression is mainly influenced by zinc.[65] Elevated expression values of this biomarker were detected in mussels transplanted in the Cetraro (1.82 Normalized Fold Expression) and Crotone (2.77 Normalized Fold Expression) study areas.

Table 3. Average of concentrations (ng/g wet weight) of chemical contaminants from the digestive gland of mussels.

Study area	Chlorpyrifos	Chlorfenvinphos	4,4'-DDE Dichlorodiphenylethylene	2,4'-DDE Dichlorodiphenylethane
Control	<0.100	<0.100	< 0.100	< 0.100
Caulonia	0.3	0.19	0.46	0.69
Cetraro	0.4	0.37	< 0.100	< 0.100
Crotone	0.186	0.22	3.224	5.776
Isola Capo Rizzuto	<0.100	<0.100	< 0.100	< 0.100
Pellaro	<0.100	<0.100	1.09	1.072
River Mesima's mouth	0.57	0.63	< 0.100	1.029
Vibo Marina	0.100	<0.100	< 0.100	2.027

The highest level of MT10 gene expression, as shown in the Crotone area, agrees with the highest concentration of zinc found by chemical analysis (145.02 µg/g) and could be ascribed to chemical contamination due to industrial activity which operated for over 50 years in the Crotone area.

The most important industrial factories were: the Pertusola South and the Montedison. The Pertusola, shut down in 1999, operated zinc sulphide producing as discarge zinc ferrites, lead, copper and cadmium. In the beginning, the Montedison produced nitrogen and phosphorus compounds to phosphoric acid generation and then, in 1993, zeolites to cleanser trade.

Cetraro study area also showed high values of MT10 mRNA expression correlated to the presence of elevated zinc value (122.12 µg/g), probably due to the mineral site composition.

Regarding the gene isoform MT20, the highest levels of expression were found in the study areas: Crotone (4.92 Normalized Fold Expression) and Isola Capo Rizzuto (2.59 Normalized Fold Expression).

While, with regard to Crotone, motivation is always to be attributed to the presence of Pertusola and Montedison (since, after the disposal, the area that housed them was never reclaimed), with regard to Isola Capo Rizzuto the motivation could be, as for Cetraro, the composition of minerals present in that area.

Furthermore, some authors, such as Lavradas *et al*. (2016), have found significant correlations between the contemporary presence of metals and metalloids in the aquatic environment and increased expression of metallothioneins in the digestive gland of mussels,[66] this could explain the high expression of MT10 at Cetraro and MT20 at Isola Capo Rizzuto. In fact, in both of these sites, the highest levels of As in the digestive glands of the studied mussels were found, this agrees with the geomorphological nature of these two places, where the rocks exhibit a high percentage of this metalloid in their composition.[67]

Many authors note that this isoform is Cd specific,[68] but in this study the concentrations of Cd found were not thought to be high enough to induce a significant response in the MT20 gene. Instead, it's plausible that the higher levels of expression found in the Crotone and Isola Capo Rizzuto areas could have been induced by a mixture of different heavy metals, which could have a synergistic effect.[69] Concerning Crotone, the increased gene expression of MT20 may depend on the increased concentration of Pb.[70] The MT20 gene response, seen in the mussels transplanted to the Isola Capo Rizzuto area, could be attributable to the elevated concentrations of Cr found in the digestive glands (7.72 µg/g).[71]

In the literature there are many evidences of increased gene expression of some metallothionein stimulated by Al, especially in the liver of mammals and in insects,[72,73] but we didn't find any studies on the correlation between Al and MT10 or MT20 in molluscs. Therefore, despite the Al levels in all stations being markedly higher than control, we must assume that the metallothioneins weighed in our study are not sensitive to its action, so they can't be considered pollutant biomarkers for this metal.

Regarding acetylcholinesterase,[74] it is amply reported in the literature that enzyme activity tends to decrease when the organism is exposed to organophosphate and carbamate compounds, used as pesticides,[75] lubricants, fuel additives.[76] Other classes of environmental contaminants, including heavy metals,[77] also determine a reduction in enzyme activity.

From the chemical data in our possession, a correlation was observed between the concentrations of organophosphate compounds considered in our study and AChE activity. In fact, at all stations the AChE activity was considerably lower than control, especially in those where the highest concentrations of organophosphate compounds were detected, specifically in Crotone, river Mesima's mouth, and Cetraro stations, where the lower levels of AChE activity were founded. Some authors reported that AChE activity can be modulated by trace metals (Cd, Cu, Hg, Zn) or natural factors (seawater temperature, biotoxins or cyanobacteria in mussel tissues).[78-81] The moderate inhibition values of AChE activity observed in Caulonia (0.0932µmoles/min^{-1}/mg^{-1}), Isola Capo Rizzuto (0.102µmoles/min^{-1}/mg^{-1}), and Pellaro (0.114 µmoles/min^{-1}/mg^{-1}) could be explained by concomitant factors of different origins, such as intensive agricultural practices in Caulonia, natural composition of the rocks in Isola Capo Rizzuto, and urban pollution (as a consequence of geographic proximity to Reggio Calabria, where is located an important port to and from Sicily) in Pellaro.

The reduction in AChE activity, in the Crotone (0.01 µmoles/min^{-1}/mg^{-1}), river Mesima's mouth (0.0199 µmoles/min^{-1}/mg^{-1}), and Cetraro (0.0349 µmoles/min^{-1}/mg^{-1}) sites could be hypothesised as the result of a synergistic effect between organophosphate compounds (Chlorpyrifos: 0.186 ng/g, 0.57 ng/g and 0.4 ng/g; Chlorfenvinfos: 0.22 ng/g, 0.63 ng/g and 0.37 ng/g respectively) and zinc levels (145.02 µg/g, 92.3 µg/g and 122,12 µg/g respectively) measured.

Enzyme activity of the cytochrome P450 isoform CYP1A1, as noted in the literature,[82] is induced by the classes of the most widely distributed contaminants, the polycyclic aromatic hydrocarbons (PAH),[83] the nitro-polycyclic aromatic hydrocarbons (NPAH),[84] the polychlorobiphenyls (PCB),[85] dioxins (TCDD)[86] and some pesticides[87] and altered by heavy metals such as Cu, Cd and Hg.[88]

The enzymatic response seen in the Crotone (2.81 pmoles/min^{-1}/mg^{-1}) and Vibo Marina (1.89 pmoles/min^{-1}/mg^{-1}) areas could be correlated with the concentration of 2,4 DDE (5.776 ng/g and 2.027 ng/g respectively), belonging to the halogenated hydrocarbons, of which the inductive effect on CYP1A1 activity is well known.[89]

The homogeneity of values measured in river Mesima's mouth (0.938 pmoles/min^{-1}/mg^{-1}), Pellaro (0.878 pmoles/min^{-1}/mg^{-1}), and Caulonia (0.812 pmoles/min^{-1}/mg^{-1}) reflected the levels of the considered contaminants (see tables 2 and 3).

Conclusions

This paper presents an overview of the significance of the use of molecular biomarkers as diagnostic and prognostic tools for marine pollution monitoring, useful for NACCP process.

Furthermore, biomarkers, inside the most recent laws in this sector (Directive 2000/60/EC, Lgs. D. 152/06 and i.s.m.), can be widely used in an integrated evaluation system of the marine environment quality. Among the various types of biomarkers, the following have received particular attention, because of their peculiar responsiveness, as extensively reported in literature: metallothioneins (MT10, MT20) expression, acetylcholinesterase activity and cytochrome CYP1A1/P450 induction. These biomarkers are being used to evaluate exposure of mussels, a sentinel marine organism, to the effect of various contaminants (organic xenobiotics and metals) using different molecular approaches (biochemical assays, fluorimetric measurement, polymerase chain reaction). The selected biomarkers indicate that the organism has been exposed to pollutants (exposure biomarkers) and that the magnitude of the organism's response is proportional to the pollutant's levels (effect biomarkers or biomarkers of stress). Results from this work give us more elements to evaluate the study areas under the bioecological impact, as prescribed by the new methodological approaches for the study of coastal marine environments. The use of biomarkers in an integrated analysis of the quality of the marine environment is by now fully accepted. This consideration, strongly recommended by the Scientific Community, should support the definitive introduction of biomarkers as routine monitoring parameters in programmes for the monitoring and control of coastal marine environments.

The identification of these biomarkers for the NACCP process,[90] applied to the marine sector, is a change of perspective in the prevention of NCDs, through a comprehensive and integrated system of information, as indicated by the vision of predictive medicine, preventive, personalized and participatory.

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Authors' Contributions

Carmela Colica, Immacolata Vecchio, and Maria Concetta Strongoli contributed equally to this project. Carmela Colica conceived and designed the experiments. Immacolata Vecchio took care of statistical data processing and the drawing of the graphs. Carmela Colica, Immacolata Vecchio, and Maria Concetta Strongoli contributed to analyzation and interpretation of the data, and to the writing and revision of the manuscript. Domenica Ventrice performed the chemical assays. Francesca Stefanizzi performed the PCR experiments. Rosario Marra, and Michelangelo Iannone provided technical support. Carmela Colica had primary responsibility for the final content. Vincenzo Mollace funded the study. All the authors read and approved the final manuscript.

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