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Application of a new targeted low density microarray and conventional biomarkers to evaluate the health status of marine mussels: A field study in Sardinian coast, Italy

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Highlights

- A targeted microarray was used to investigate gene expression in mussels
- biomarker data were in trend with transcriptomic output
- Data were integrated by the MES, defining the area in which the presence of chemicals is toxic.
- The integration of transcriptomic and biomarker data: a promising biomonitoring programs strategy.

Abstract

In the present study, we investigated the health status of marine mussels (*Mytilus galloprovincialis*) caged and deployed at three different sites on the Sardinian coast characterized by different levels of contamination: Fornelli (F, the reference site), Cala Reale (CR), and Porto Torres (PT). A new low density oligonucleotide microarray was used to investigate global gene expression in the digestive gland of mussels. Target genes were selected to cover most of the biological processes involved in the stress response in bivalve mollusks (e.g. DNA metabolism, translation, immune response, cytoskeleton organization). A battery of classical biomarkers was also employed to complement the gene expression analyses. Chemical analysis revealed higher loads of heavy metals (Pb and Cu) and total polycyclic aromatic hydrocarbons (PAHs) at PT compared to the other sites. In mussels deployed at CR, functional genomics analysis of the microarray data rendered 78 differentially expressed genes (DEGs) involved in 11 biological processes. Animals exposed at PT had 105 DEGs that were characterized by the regulation of 14 biological processes, including mitochondrial activity, adhesion to substrate, DNA metabolism, translation, metal resistance, and cytoskeleton organization. Biomarker data (lysosomal membrane stability, lysosomal/cytoplasm volume ratio, lipofuscin accumulation, metallothionein content, micronucleus frequency, and cytoskeleton alteration) were in trend with transcriptomic output. Biomarker data were integrated using the Mussel Expert System (MES), allowing defining the area in which the presence of chemicals is toxic for mussels. Our study provides the opportunity to adopt a new approach of integrating transcriptomic (microarray) results with classical biomarkers to assess the impact of pollutants on marine mussels in biomonitoring programs.

Keywords: biomarkers; targeted microarray; mussels; data integration.

Introduction

Organisms are the ultimate target for anthropogenic toxicants-induced damage in a complex environment. Therefore, our conventional reliance on analytical tools is not sufficient to evaluate ecosystem health. Sub-lethal biological changes or biomarkers are consequently also considered to be relevant tools for environmental risk assessments (Dallas and Jha, 2015). In this context, *Bivalve mollusks* (i.e. *Mytilus* spp.) have been established as one of the most widely used organisms for environmental monitoring. Mussels are particularly useful in this context because they are sessile, occupy regions of different pollution levels, and may accumulate both organic and inorganic pollutants in their tissues (Viarengo et al., 2007; Bayne, 2009; Al-Subiai et al., 2011). Evidence of a stress syndrome and the biological risk associated with contaminated environments have been established by the use of a set of physiological and cellular markers, (Bouraoui et al., 2010). However, our knowledge of bivalve physiology is still limited, as well as how mussels respond to complex mixtures of contaminants that are often present in the marine coastal environment.

Biomarkers can be very useful to determine the organism's stress response to individual toxicants or their mixtures (Depledge and Fossi, 1994; Moore et al., 2012; Bebianno and Minier, 2014). Lysosomal membrane stability (LMS), lysosomal/cytoplasmic(L/C) volume ratio, lipid peroxidation products (malondialdehyde content, protein carbonylation level, lipofuscin [LF] lysosomal accumulation), antioxidant enzyme activities, DNA damage, and micronucleus frequency measurements are frequently used as sensitive tests in mussels to estimate the cytotoxic and genotoxic effects of chemicals (Banni et al., 2009; Bolognesi et al., 1999; Moore et al., 2007; Viarengo et al., 2007; Dallas et al. 2013; Dallas and Jha, 2015; Di et al., 2011, 2017).

A set of sub-lethal biological responses or biomarkers were standardized to cover the possible biological effects of a specific class of contaminants, such as the use of metallothionein (MT)

content to assess exposure to heavy metals (Viarengo et al., 1997) and the inhibition of acetylcholinesterase activity to monitor the effects of insecticides (Narbonne et al., 2005). However, as mentioned above, it is crucial to also study animal's overall health status using markers of stress suitable for highlighting potential pathological alterations of the organism due to the total charge of pollutants.

Genomics-based approaches have provided insight into the mechanisms underlying suites of biological processes. Particularly, transcriptomics, the evaluation of mRNAs abundances in a biological sample, has been a robust tool, enhancing our knowledge of many relevant physiological processes in marine organisms. Recently, our research group developed a new low density and low cost oligonucleotide microarray for mussel species (Banni et al., 2017). Target genes employed in the new platform were selected to cover most of the relevant biological processes. These included DNA metabolism, immune response, translation, oxidative stress, heat shock response, biotransformation, endocrine response and reproduction, mitochondrial activity, carbohydrate metabolism, cytoskeleton organization, proteolysis, ion channels and transporters, lysosomal activity, metal homeostasis, adhesion to substrate as a result of stress response in bivalve species.

The current investigation aimed to integrate the transcriptomic approach through the use of this newly developed low density array complementing other classical biomarker responses in an ecotoxicological context. Three sites along the Sardinia coast characterized by different levels of contamination were investigated using caged mussels as sentinel organisms deployed at these sites. Firstly, we determined the bioaccumulation of metals (i.e. Cd, Pb, Hg, and Cu), as well as total PAHs, in the mussel's digestive gland; in the same tissue, PAHs accumulation and subcellular distribution were investigated by immunofluorescent labeling. Secondly, transcriptomic evaluation of the differentially expressed genes (DEGs) was achieved using our recently developed platform. Finally, a set of biomarkers at the cellular and tissue level,

including LMS, L/C volume ratio, LF lysosomal content, tubulin organization, and MTcontent, were measured; the genotoxic effects were studied by evaluating the micronucleus (MN) frequency. The biomarker data were then integrated by the Mussel Expert System (MES) to objectively rank the level of stress syndrome in mussels.

Material and Methods

Sardinia samples and sampling sites

Mussels (*M. galloprovincialis* Lam.), 4-5 cm in length, were obtained from a farm in Arborea (OR, Sardinia, Italy) and kept in cages (polypropylene mesh bags) and transplanted (240 mussels per site) for 28 days (October-November 2013) at three sites (Fig.1) along the Sardinian coast. The transplantation sites included: Fornelli (F; 40°59'32.1"N 8°12'54.5"E,reference site), CalaReale (CR; 41°03'42.7"N 8°17'17.5"E,a small marina) and Porto Torres (PT; 40°50'23.1"N 8°24'16.9"E,large industrial and commercial seaport). Mussels caged in polypropylene mesh bags were placed approximately 4 m under the water. At the end of the deployment period, mussel sex was determined by mantle smear and light microscopy. Digestive glands from females were processed for transcriptomics (microarray and qRT-PCR) and immunohistochemical studies (Banni et al., 2017). Individuals of both sexes were used for the assessment of genotoxicity (MN frequency) in gills (Bolognesi et al., 1999; Dallas et al., 2013). Tissue samples were also taken for chemical analysis, transferred into pre-weighed glass vials, and frozen for storage prior to analysis.

Chemical analyses

Metals (Cd, Cu, Hg, Pb) were determined by ICP-MS technique after acid digestion of samples in a microwave oven CEM Discover SP-D (CEM Corp., United States). Analysis were performed in application of EPA 3052 method (USEPA, 1996a) for sample treatment, and EPA

6020A (USEPA, 1998) for instrumental analysis. PAHs were determined by UPLC-FLD technique after ASE extraction and SPE cleanup (USEPA, 1996b, 1996c). The analyses were carried out in the accredited Official Appointed Lab of Sassari, under QA/QC schemes provided by the EU legislation.

Microarray hybridization and analysis

Competitive dual-color microarray hybridization was performed using the new STREM platform (Banni et al., 2017). The procedure was performed as described previously (Banni et al., 2011) using 0.5 µg of an anchored oligodT(19)VN. Total RNA was extracted from individual digestive glands using acid phenol-chloroform according to (Chomczynski and Sacchi., 1987) and TRI-Reagent (Sigma-Aldrich). The RNA quality was checked by UV spectroscopy and TBE agarose gel electrophoresis (Negri et al., 2013). Laser scanning of microarrays was obtained using an Agilent G2565CA scanner (Agilent Technologies, Inc., USA) at 5-µm resolution. Sixteen-bit TIFF images were processed using Genepix 6.0 (Axon) to extract raw fluorescence data from each spot.

Global mean normalization and Log₂ transformation was performed for each expression level as described in Banni et al., (2017). Each experimental condition was calculated as 12 different values (4 biological replicates, pool of at least 3 animals and 3 technical replicates). DEGs were identified by Significance Analysis of Microarray (SAM, <http://statweb.stanford.edu/~tibs/SAM/>).

The experimental design accounted for a complete “triangular loop” in which each RNA sample from the tissue taken from mussels caged in PT and CR was hybridized with RNA from control mussels (mussels caged in F). MIAMI-compliant microarray data, including a detailed description of the experimental design and each hybridization experiment (24 experiments),

were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/>) under identifier GSE97099.

Application of qRT-PCR

The qRT-PCR reactions were carried out using the same RNA extract as microarray hybridization. Seven probes and primer pairs (mt10, mt20, cat, gst, sod, hsp70, and tubulin; Table S1) were used. All primers and dual-labeled TaqMan probes were synthesized by MWG-Biotech GmbH (Germany).

The cDNA preparation and the Q-PCR details are as described in Banni et al., (2011), Negri et al., (2013) and Banni et al., (2017). The relative expression data were normalized geometrically to an invariant actin isotype (AJ625116), ribosomal protein riboL27 (AJ625928) and 18S rRNA (L33452). Statistical analyses were carried out on the group mean values using a random reallocation test (Pfaffl et al., 2002).

Biomarker analysis

A battery of biomarkers was used to evaluate the biological effects induced in mussels after transplantation (caging) for 28 days at the different field sites, mentioned earlier. The determination of LMS and L/C volume ratio and LF lysosomal content were performed (n=10) as described respectively by Moore (1988), Moore and Clarke,(1982), Pearse, (1972) and Moore, (1988).

Metallothioneins (MTs) content was evaluated in digestive glands (n=10) according to the spectrophotometric method described in Viarengo et al. (1997) based on cysteine residues titration of a partially purified metallothionein extract.

The micronucleus (MN) assay was performed in gill cells from mussels (n=10) caged at the different sites following the method described elsewhere in details (Bolognesi et al. 1999; Dallas et al., 2013).

Immunofluorescence studies

Frozen digestive gland sections (10 µm) obtained using a cryostat as described above were flash-dried by transferring them onto poly-L-lysine-coated microscope slides at room temperature and fixed in paraformaldehyde (PFA) solution (4% PFA in phosphate buffered saline[PBS], pH 7.2) for 20 min at $20 \pm 1^\circ\text{C}$.

Immunofluorescent anti-PAH staining was carried out (n=5) on frozen digestive gland sections (10 µm) obtained using a cryostat as described by Sforzini et al.(2014) and Banni et al., (2017).

Immunofluorescent anti-tubulin staining was performed as described by Banni et al al.(2015) and Banni et al., (2017).

Univariate statistical analysis

Experiments were performed in at least five replicates and statistical analysis carried out using the non-parametric Mann-Whitney U-test ($p<0.05$).

Mussel Expert System (MES)

Biomarker data obtained from mussels deployed in CR and PT were compared with data from mussels exposed at the reference site (F). Only significant changes >20% were utilized to evaluate alterations in the health status of the organisms. The algorithm applied in the integration procedure was used to generate an Expert System of Classification, software that is now available at the DiSIT website (http://disav.unipmn.it/index.php?page=expert-system&hl=it_IT)(Dagnino et al., 2007;El Haimeuret al., 2017).

Results

Contaminants in tissue samples

After 28 days of transplanted to different sites, caging, mussels sampled at PT site presented the highest heavy metal accumulation (Table 1) in terms of Pb, Cd, Hg and Cu (0.4, 0.14, 0.03 and 12.50 mg/kg dry weight, respectively). Mussels caged at PT had the highest total PAHs content, with up to 3.30 $\mu\text{g}/\text{Kg}$ dry weight.

Immunohistochemical analysis highlighted a high amount of pyrene-based PAHs in the digestive gland cells from mussels caged at PT; the fluorescence was very low in animals caged at CR, a small marina characterized by a low level of contamination (Table 1).

Microarray data

Dual-color hybridization revealed evident alterations in gene expression patterns in the investigated tissues due to the site exposure (Fig 2, Fig. 3, and Table S2, Table S3). Our data revealed distinct patterns for 142 DEGs in the digestive gland tissues.

As our new platform is based on annotated genes that are already classified into well-known biological processes (Table S2), we generated the list of biological processes involved in the mussel's response to stress depending on the field sites along the Sardinian coast from the DEGs obtained for each condition (Fig. 4 and 5). Our data highlighted 11 contributing biological processes in the digestive glands of mussels caged for 28 days at CR. The response was dominated largely by up-regulation of translation, cytoskeleton organization, carbohydrate metabolism, and immune response-related genes (Fig. 4, Table S3).

The main contributing biological processes in the mussel's response to PT conditions are reported in Fig. 5 and Table S3. Our data indicate that immune response, mitochondrial activities, adhesion to substrate, DNA metabolism, metal resistance, and cytoskeleton are the most dominating processes.

Confirmation analysis

We carried out qRT-PCR to confirm and refine the relative expression levels of six homologous genes belonging to the most important biological processes, including the genes encoding tubulin (cytoskeleton), Zn-Cu superoxide-dismutase and catalase (oxidative stress), MT isoforms mt-10 and mt-20 (metal homeostasis), and gst (phase II biotransformation). Microarray and qRT-PCR data indicated a positive relationship in all cases (Fig.6). Indeed, catalase (cat), superoxide dismutase(sod), and glutathione-s-transferase (gst) were positively regulated in animals caged at PT (1.91, 2.72, and 2.63-fold compared to control, respectively). The two MT cognates were also significantly up-regulated at PT (3.44 and 5.62-fold compared to control, respectively). Finally, the tubulin- and hsp70-related targets were significantly up-regulated in animals caged at the two locations.

Biomarker responses

The biomarkers in digestive glands from mussels transplanted for 28 days along the Sardinian coast highlighted significant alterations at PT with respect to F (reference site), without mortality (data not shown). Fig.7 A, B shows the effects on the LMS and L/C volume ratio of the digestive gland cells. Though the LMS was significantly affected in animals caged at CR and PT compared to the reference site, the L/C volume ratio was only significantly affected at PT(+89% with respect to F). This result indicates a loss of cytoplasm due to enhanced lysosomal activity in the digestive gland tissue of mussel's caged at PT.

Fig.7 C shows the LF accumulation in digestive gland cells from caged mussels. A significant increase in LF accumulation was observed in the organisms caged at CR and PT (+94% and +99%, respectively) compared to the reference site.

Total MT content was also evaluated in the digestive glands (Fig. 7 D). A significant increase in MT levels with respect to the reference site was observed for PT (104 µg/g fresh tissue), whereas mussels caged at CR had MT concentrations similar to those measured in controls.

The possible effects on tubulin, which increased in mRNA content in mussels caged at CR and PT, were evaluated using an immunohistochemical approach. The amount of tubulin in the digestive gland cells increased in mussels from CR, reaching a maximum in the animals from PT (Fig. 7 E). In these animals, the cytoplasm of the cells contained numerous highly fluorescent granules that contribute to determining the observed increase in tubulin (Fig. 7 E, image B).

Finally, the genotoxic effects were evaluated in gill cells from transplanted mussels using the MN test (Fig. 6 F). No significant effect was observed in mussels caged at CR. However, mollusks exposed at PT exhibited a significant increase in MN frequency compared to the reference site (4.2%).

Data integration by the MES

The biomarker data obtained from mussels caged along the Sardinian coast for 28 days were integrated using the MES to obtain an objective assessment of the stress level experienced by the organisms (Fig. 8). The system classified the mussels exposed at CR as being subjected to “medium stress” (C level), as these animals exhibited alterations in only the LMS and LF accumulation. Mussels caged at PT, in which we observed alterations of biomarkers at the molecular/cellular level (i.e., LMS, LF accumulation, and MN frequency), as well as at the tissue level (L/C volume ratio), were ranked as being subjected to “high stress” (D level).

Discussion

The quality of a marine coastal environment can be assessed using a set of methods combining both chemical and biological data (El haimeuret al., 2017; Dallas and Jha, 2015). Numerous early warning biomarkers have been utilized and applied to identify and quantify potential alterations in the health status of organisms living in marine ecosystems (Moore et al., 2012). The integration of molecular techniques, such as the evaluation of expression levels of specific target genes (Banni et al., 2007) or the use of microarray, with biomarkers at different levels of functional complexity and chemical data could may provide more information about the real physiological and fitness status of the organisms.

In this study, chemical analyses of the samples demonstrated distinct levels of contamination in terms of heavy metals and PAHs in mussels transplanted in the harbor area of PT, a site with relatively high pollution compared to animals transplanted at CR, a small marina. The mussels sampled from F, which was considered a reference site due to the minimal anthropogenic contamination, presented the lowest chemical load. Immunohistochemical data obtained by recent novel methodology (Sforzini et al., 2014) demonstrated a higher PAH load in digestive gland tissues from mussels transplanted at PT and showed the distribution of these chemicals in the cells. In a recent study, pyrene-based PAHs accumulated in vesicles that were recognized as part of the lysosomal vacuolar system (Sforzini et al unpublished).

A new low density oligonucleotide microarray was employed to study alterations in digestive gland gene expression profiles in mussels caged for 28 days at the three sites along the Sardinian coast. In this study digestive gland was used as the most metabolically active organ. Moreover, Banni et al., (2011) highlighted importance difference in term of gene expression regulation between the two sexes in mussels, thus sex was fixed for transcriptomic analysis. Interesting information was deduced from the transcriptomic data. The number of DEGs was greater in mussels from PT (n=105) than in mussels from CR (n=78), with a total of 142 DEGs in at least

one of the two conditions. Among the processes depicted in the more pronounced mussel response at PT than CR are translation, mitochondrial activity, cytoskeleton organization, carbohydrate metabolism, and DNA metabolism.

Most of the regulated DEGs related to the translation process encode ribosomal protein subunits (Table S3). This regulation may indicate that mRNA-directed protein synthesis is affected in mussels exposed to higher PAH and metal loads (Maria et al., 2013; Negri et al., 2013; Banni et al., 2017). PAHs and toxic metals are known to interfere with protein synthesis, protein degradation, ATP production, and structural protein changes in response to stress in *M. galloprovincialis* (Farkas et al., 2015). In addition, ribosome biogenesis in bacteria and zebrafish has been reported to be modulated by environmental stressors (Al Refaii and Alix, 2009; Connolly Hall, 2008).

Mitochondrial activity and carbohydrate metabolism are among the processes that contribute to the response in mussels caged at CR and PT. Recently, our research group (Banni et al., 2017) highlighted the implication of both processes in the mussel's response to increasing concentrations of B[a]P in two metabolically different tissues, the digestive gland and gills. These changes in mitochondria- and carbohydrate-associated genes are particularly relevant in terms of respiration processes through which energy is provided to the cells. Moreover, anaerobic ATP synthesis could be important in mollusks (Bayne, 2009). In fact, this bivalve tends to lock its valves in the presence of polluted water, reducing the respiration rate (Connor et al., 2016), which could lead to a need for the organism to increase anaerobic ATP production. Cytoskeleton organization was previously reported to be one of the main targets of xenobiotics and environmental stressors, such as thermo-tolerance and higher PAH loads, as well as PCBs (Banni et al., 2016, 2017). Thus, mussels may regulate cytoskeleton reorganization and repair to cope with increased stress. Induction of α -tubulin in B[a]P-exposed mussels was recently

proposed to be a key event in the maintenance of microtubule organization and vesicular movement (Maria et al., 2013).

We recently used an immunohistochemical approach to assess tubulin and actin organization and accumulation in mollusk cells (Banni et al., 2016, 2017). In this study, our results showed a particularly relevant increase in tubulin-related fluorescence in the digestive gland cells of mussels caged for 28 days at PT; the enhancement of fluorescence was due mainly to its presence in cells with tubulin-rich granules. The formation of granules containing tubulin has been described in cells from different organisms as a consequence of few stress conditions (Clark and Shay, 1981; Martin et al., 2010; Banni et al., 2016, 2017). The damaged tubulin could be compartmentalized in vesicles, and the decrease in free tubulin protein should stimulate tubulin neo-synthesis (Clark and Shay, 1981; Martin et al., 2010) through enhancement of transcription in an attempt to maintain the cytoskeletal architecture necessary for cell functions, such as vesicle transport and autophagy. These data are consistent with the increased expression of tubulin-related genes in mussels from PT compared to control. Similarly, Banni et al. (2017) reported that increasing concentrations of B[a]P is marked by the up-regulation of genes encoding proteins involved in cytoskeleton maintenance and repair.

DNA repair processes involved four DEGs at CR and 10 at PT. The gene expression levels of associated genes (Table S3) may indicate the occurrence of a DNA repair event in mussel tissues that was more pronounced at PT, as indicated by the expression levels of DNA ligase and topoisomerase. Mussel's cells may respond to DNA damages through p53-mediated cell cycle arrest or apoptosis (Banni et al., 2009; Di et al., 2011, 2017). Moreover, bulky B[a]P-DNA adducts are reportedly removed by nucleotide excision repair (NER) enzymes, including DNA ligase (Hess et al., 1997). Topoisomerase, a DNA replication/repair-related enzyme, has been implicated in mammalian cells exposed to polycyclic aromatic hydrocarbons (Yakovleva et al., 2006).

In the present study, the DNA stability was investigated in gill cells. Due to their rapid turnover, these epithelial cells are useful for studying MN formation, an event that depends on the breakage or loss of chromosomal DNA during cell division (Widdows et al., 2002). Genotoxic damage was evident after the caging period in the mussels sampled from PT. Cellular alterations were probably caused by exposure to both metals and PAHs present in the water as a result of the up-take and metabolic transformation of organic chemicals. The genotoxic effects of Cd and Pb (Varotto et al., 2013) and PAHs (Akcha et al., 2000;Kamel et al., 2012) have been well documented in *Mytilus sp.* using several techniques, including MN and Comet assays. In addition to the direct genotoxic effect of chemicals and their metabolites, the production of reactive oxygen species is likely to be a key mechanism in genotoxicity, and this can lead to DNA alteration (Barillet et al., 2010; Dallas et al., 2013?). Thus, the observed levels of LF, a lipid peroxidation end product increased in digestive gland cells from mussels at PT, may explain, at least in part, the genotoxic effects observed in these caged organisms.

In this study, metal resistance was among the biological processes depicted only in mussels from PT. The gene transcription analysis demonstrated that both MT cognates, the mt-10 and mt20 genes, were significantly up-regulated in mussels from PT, reflecting the biological response of mussels to heavy metal contamination.

MTs have been used largely as biomarkers response to exposure to heavy metal contamination under field conditions, in both fresh and sea waters (Tlili et al., 2010; Banni et al., 2007). A common practice for use of this parameter in biomonitoring exercises has been the assessment of the total MT protein content in a partially purified cytosolic extract (Viarengo et al., 1997). In mussels, the first report of the use of mt-10 and mt-20gene expression levels in addition to the MT protein content in a field context was by Banni et al.(2007), allowing a better understanding of the mechanism by which these proteins defend the cells against metal induced toxicity. In this study, the transcriptional data from micro-array and qRT-PCR confirmation

were in trend with the biomarker response. A substantial increase of the mt-10 and mt-20 targets in PT was concomitant with the increase in metalloprotein in the digestive glands of mussels from this site.

Heavy metals may stimulate ROS production and can interfere with the activity of antioxidant enzymes, such as catalase and superoxide dismutase. This interference can lead to oxidative stress in the cells (Tran et al., 2007; Viarengo et al., 2007). PAHs are also known to enhance ROS productions in the cells (Akcha et al., 2000; Kamel et al., 2012). As mentioned above, peroxidation products, such as LF, were observed in digestive gland cells from mussels caged in contaminated sites, reaching the maximal level at PT. Digestive gland tissue is the “metabolic” tissue of mussels. The increased level of oxidative stress in this tissue therefore may help explain, at least in part, the toxicity of the contaminants present in the water.

Caging of the organisms at the impacted sites significantly affected the LMS of digestive gland cells. The LMS significantly decreased in mussels caged at CR with respect to F; this decrease was very pronounced in mussels caged at PT. The lysosomal system appears to be a common target for many environmental contaminants, as lysosomes accumulate many toxic metals and organic xenobiotics, which may alter normal function and disturb the lysosomal membrane (Moore et al., 2008). Therefore, it is very important to assess the LMS, as this subcellular pathological response represents an extremely sensitive general index of health and may be the first sign of the toxic effects of pollutants in different organisms (Viarengo, 1989; Köhler et al., 2002; Moore et al., 2012). LMS is now a mandatory biomarker in the UnepMap biomonitoring program in the Mediterranean Sea. Our data are in agreement with the data reported by Kamel et al. (2012) and Negri et al. (2013) for the digestive glands of mussels exposed to the PAH B[a]P and copper, respectively.

Lysosomal membrane destabilization indicates increased autophagic activity. Stressed cells affected by toxic chemicals react by degrading the damaged cellular macromolecules and

organelles. If the increased activity of the lysosomal vacuole system is balanced by an increase in the rate of protein synthesis, the result is increased turnover of the damaged cellular components. Thus, the most important effect of xenobiotics is higher energy expenditure. However, if the contaminant level is so high that it affects protein synthesis, the cells enter a “catabolic” status (i.e., they start to lose part of their cytoplasm due to unbalanced autophagic activity). An increase in the LC volume ratio is a sign of such a situation. In the present study, this ratio was enhanced in animals caged at PT. These results may indicate an alteration in the growth of mussels in this area in particular and in highly polluted zones in general. The LMS correlates with the scope for growth (Moore et al., 2006); LMS is now considered in different model organisms to be a prognostic biomarker capable of providing early evidence of future noxious consequences at the population level (Moore et al., 2006; Sforzini et al., 2018). A possible interpretation of these results involves the role of mechanistic target of rapamycin (mTOR), a key element in the regulation of autophagy, protein synthesis, and energy metabolism (Antikainen et al., 2017). In mussels exposed to toxic chemicals, the level of the phosphorylated active form of mTOR was recently demonstrated to decrease strongly; in the same animals, a drastic increase in autophagy was observed (Sforzini et al., 2018).

Finally, the biomarker data were integrated with the MES (Dagnino et al., 2007; El haimour et al., 2017) to objectively rank the level of stress experienced by mussels caged along the Sardinian coast. The bioavailable metals and PAHs (Table 1) present at PT, the most contaminated site, negatively affected the caged mussels, which exhibited signs of high stress (D level). Although the level of contamination at CR (a small marina located in the protected area of the Asinara island) was lower than at PT (an industrial harbor), mussels caged at CR exhibited a medium level of stress (C level) due to a decrease in the LMS and increase in oxidative damage. Therefore, use of the MES allows numerous complex biological responses to be integrated to give an objective determination of the health status of the mollusks; this

information is fundamental for determining the extent of the area in which contamination reduces the quality of marine waters becoming toxic for filter feeding organisms.

In conclusion, the changes in the transcriptomic profiles of digestive glands from mussels sampled at three sites were found to follow the noxious effects of increasing contamination of the organisms. The gene expression data obtained using the new low density microarray showed a clear trend not only in terms of the number of genes involved in the mussels' response to stress, but also in the extent of gene expression. In depth laboratory-based investigations should be performed to link transcriptomic outputs with the level of stress syndrome in the organisms. Wide variability in biomarker responses is often cited as a reason for their reluctant use in regulatory frameworks. However, there are several approaches that have been recommended to promote the adoption of biological responses as a tool and minimise observed variability (Dallas and Jha, 2015). Our results highlight a close relationship between the genomic results and changes in cell functions as depicted by the biomarker responses; these parameters are essential tools for the evaluation of marine environmental quality, as they are responsive only to the bioavailable fraction of contaminants. Moreover, immunohistochemical detection of PAHs was suitable for highlighting the tissue and subcellular distribution of minimal amounts of pyrene-based PAHs. The integration of biomarker responses by MES may represent a tool by which environmental managers can interpret the complex biological data to evaluate the health status of the organisms and define areas that should be a priority for protecting ecosystem quality, ecosystem services and eventually decontamination. Although there are many challenges (Snape et al., 2004; Thomas and Klaper, 2004), mechanistic information from microarray studies (and other 'omic' approaches) allows us to build strong foundations on which to base other biomarkers (Dallas and Jha, 2015). Such an approach may also be a good starting point to develop remediation methods or treatments for environmental health issues, as

is the case for medical microarrays in the human health arena. Our study goes some way towards achieving these broader goals.

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Table 1: Chemical analysis of mussel tissues following transplant exposure for 28 days in Fornelli, Cala-Real and Porto Tores, Sardinia, Italy.

Sites	Heavy metals (mg/kg)				PAHs ($\mu\text{g/kg}$)
	Pb	Cd	Hg	Cu	
Fornelli	0.20 \pm 0.011	0.10 \pm 0.07	0.001 \pm 0.0009	0.50 \pm 0.023	0.14 \pm 0.08
Cala Real	0.32 \pm 0.015*	0.13 \pm 0.09*	0.09 \pm 0.05*	0.66 \pm 0.029*	0.22 \pm 0.013*
Porto Tores	0.40 \pm 0.016*	0.14 \pm 0.09*	0.03 \pm 0.01*	12.5 \pm 0.087*	3.30 \pm 0.16*

* indicates statistically significant differences with respect to Fornelli (considered as reference sites) (n =15, $p < 0.05$ Mann-Whitney U -test).

Table 2: Number of DEGs depicted in animals caged for 30 days in Cala-Real and Porto Tores against controls (Caged for 30 days in Fornelli). Shown are total DEGs, number of up-regulated and Down-regulated DEGs.

	Cala Real	Porto-Tores
Total DEGs	78	105
Up-regulated	54	63
Down-regulated	24	42

Fig 1. Study areas along the Sardinia coast indicating locations of the stations for mussel transplants for 28 days.

Fig 2. Quantitative fluorescence analysis of anti-PAHs immunoreactions of digestive gland tissue sections from mussels transplanted at different sites. Data represent the mean \pm SD of at least five replicates. * indicates statistically significant differences ($p < 0.05$ Mann-Whitney *U*-test). Representative images of tissue sections of mussels from Fornelli (Reference site) (A) and Porto Torres (B) (green: FITC conjugated secondary antibody).

Fig 3: *Mytilus galloprovincialis* gene expression profiles of digestive gland tissue in animals caged for 28 days in Cala Real (CR) and Porto Torres (PT). The heat map (A) (Pearson correlation, complete linkage algorithm) reports the log₂ relative expression level with respect to Fornelli. 142 differentially expressed genes were generated in at least one condition. Supporting information to Figure 2 is presented in Table S2 and Table S3. Venn diagram representation of gene expression patterns (Panel B) clearly depicted that only 14 differentially expressed genes (DEGs) are shared between the two sites. All DEGs are obtained with respect to the control condition (mussels caged at Fornelli site). Data used to create the Venn-diagram were obtained from microarray analysis (Table S3).

Fig 4: Over-representation analysis of DEGs in the digestive gland of animals caged at Cala Real (CR). The figure shows: experimental condition; biological processes; Number of up-regulated genes; Number of down-regulated genes. The over-represented biological processes in exposed animals versus control (mussels transplanted at Fornelli site). (Table S3)

Fig 5: Over-representation analysis of DEGs in the digestive gland of animals caged at Porto Torres (PT). Showed are: experimental condition; biological processes; Number of up-regulated genes; Number of down-regulated genes. The over-represented biological processes in exposed animals versus control (mussels caged in Fornelli site). (Table S4)

Figure 6: QPCR data of 7 selected targets. Gene expression was performed respect to 18°C and was normalized against Actin, 18S and Ribo L27. * Significantly different from reference condition (Fornelli), a: Significantly different from Cala * $p < 0.05$ threshold cycle random reallocation test according to Pfaffel et al. (2002) $n = 4$.

Fig 6. Biological responses in mussels caged at different sites. (A) Lysosomal membrane stability (LMS); (B) lysosomal/cytoplasmic volume ratio (L/C); (C) lipofuscin content (LF); (D) metallothionein content (MT); E) tubulin level (quantitative fluorescence analysis of anti-tubulin immune-reaction. A, B) Representative images of tissue sections of mussels from Fornelli (Reference site) (A) and Porto Torres (B) -red: Goat Anti-Rabbit IgG H&L (DyLight® 594-); F) micronuclei (MN) frequency. Data represent the mean \pm SD of at least five replicates. * indicates statistically significant differences ($p < 0.05$ Mann-Whitney *U*-test). (A-E were evaluated in digestive gland; F in gill cells).

Fig 7. Stress level in mussels caged at different sites obtained by integrating biomarker responses with MES.

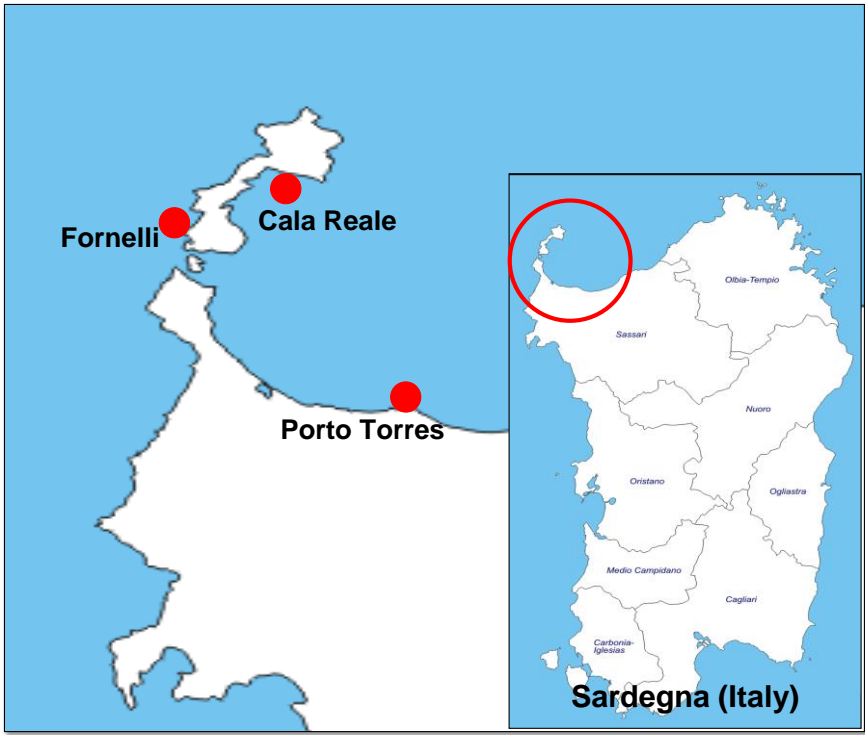


Fig. 1.

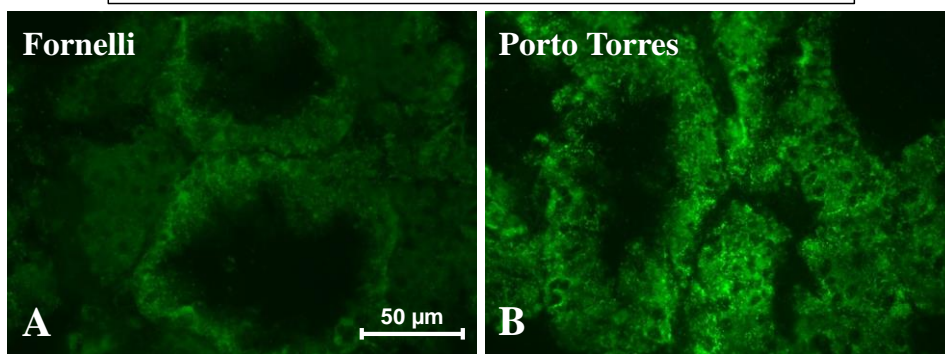
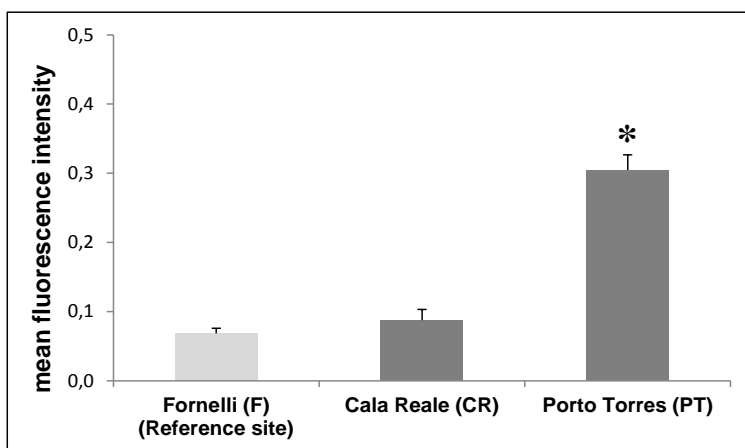


Figure 2.

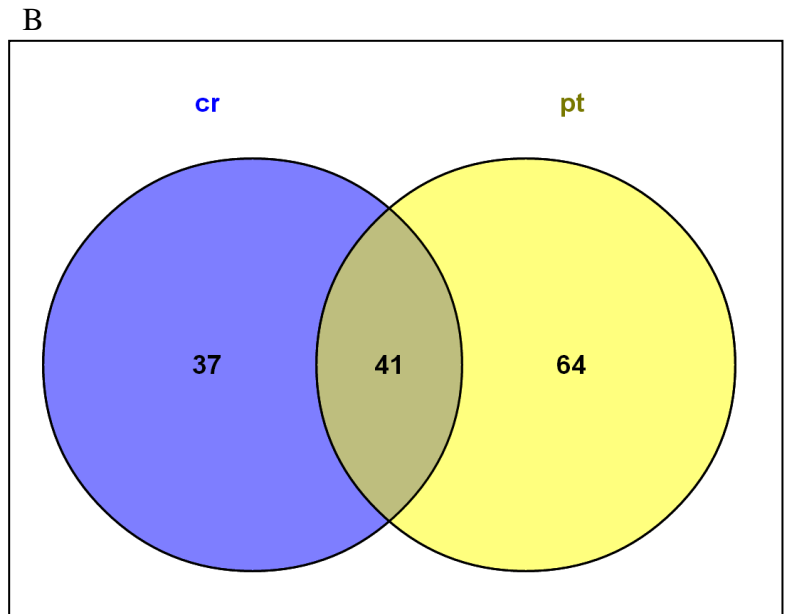
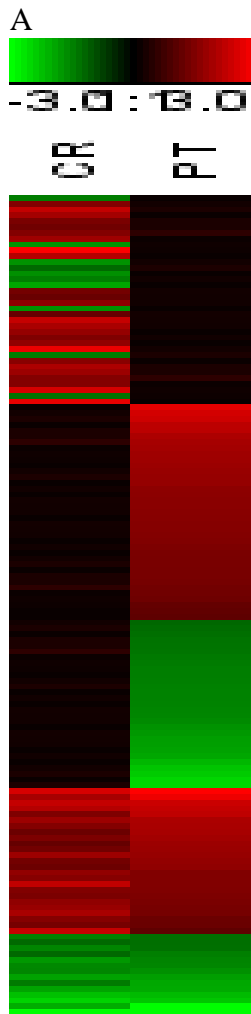


Figure 3

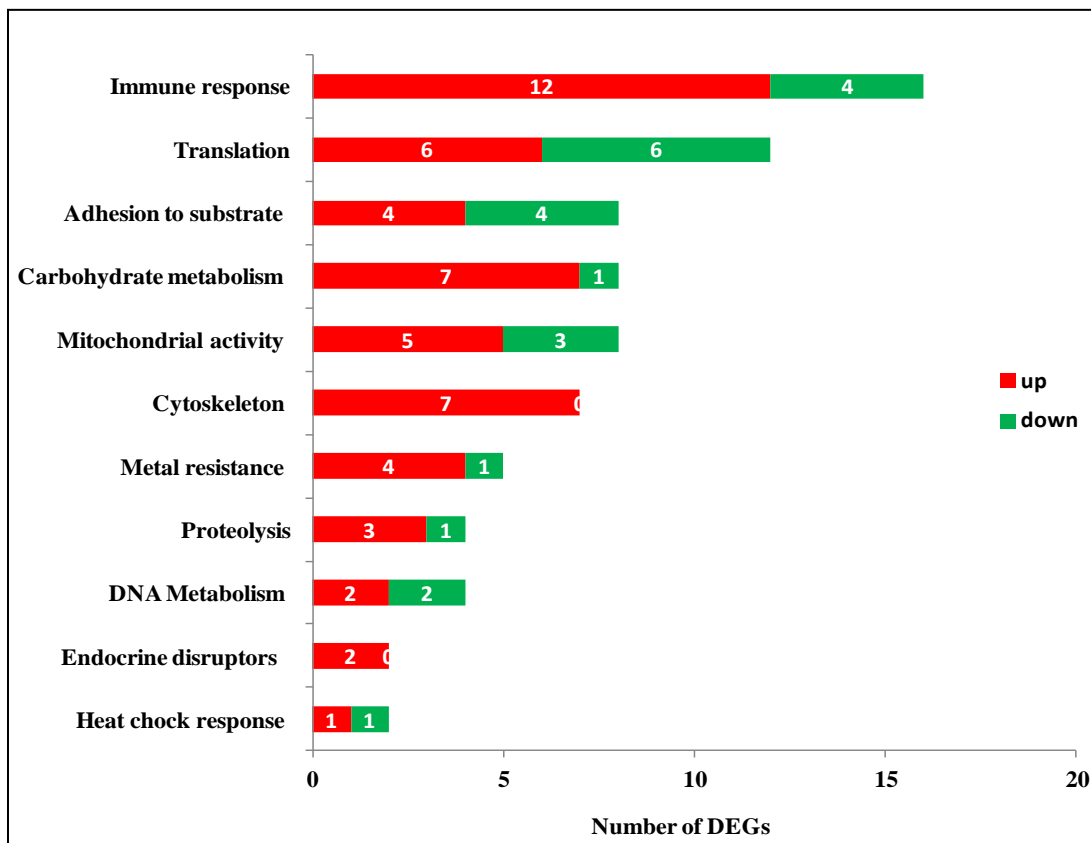


Figure 4

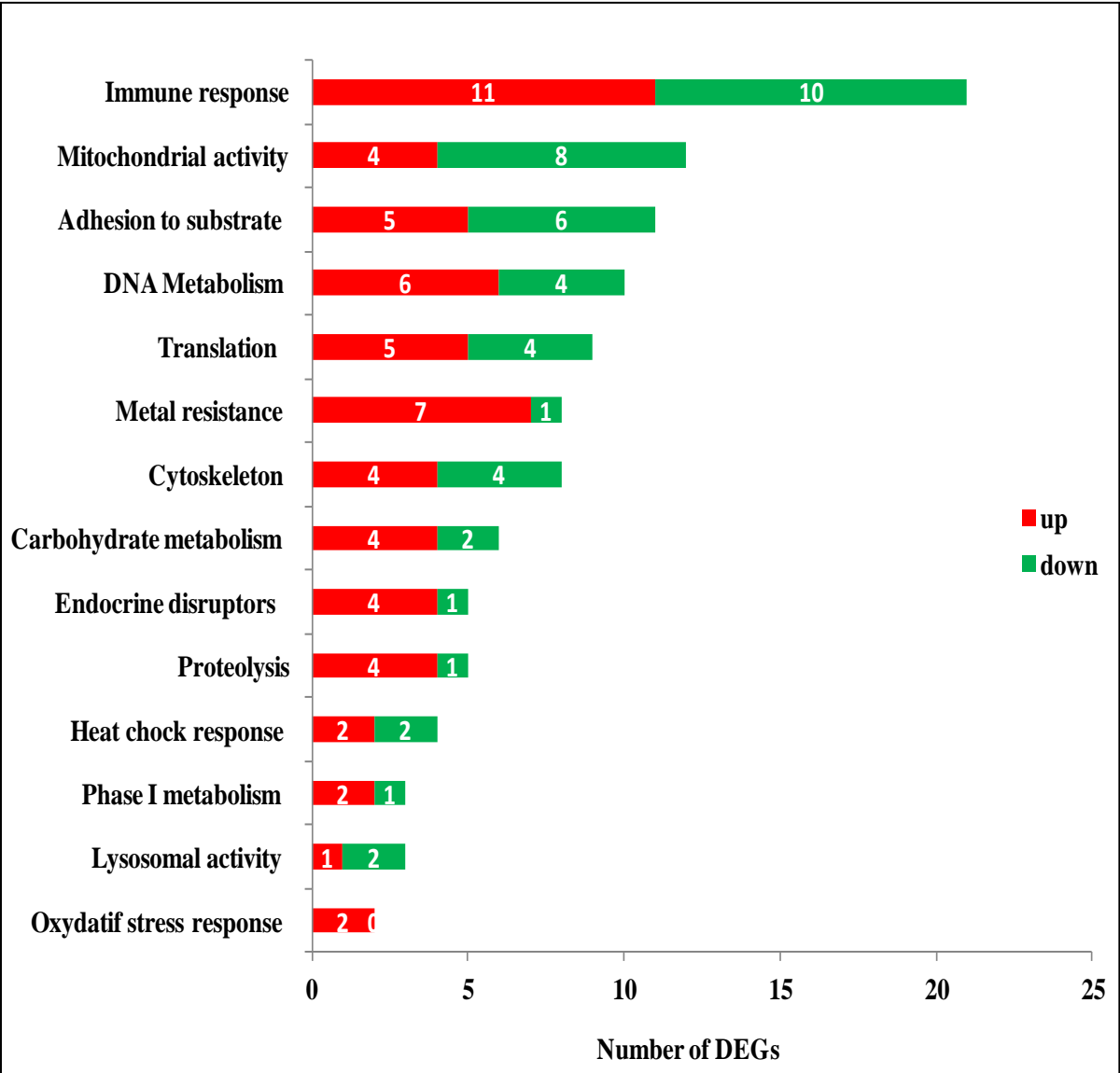


Figure 5

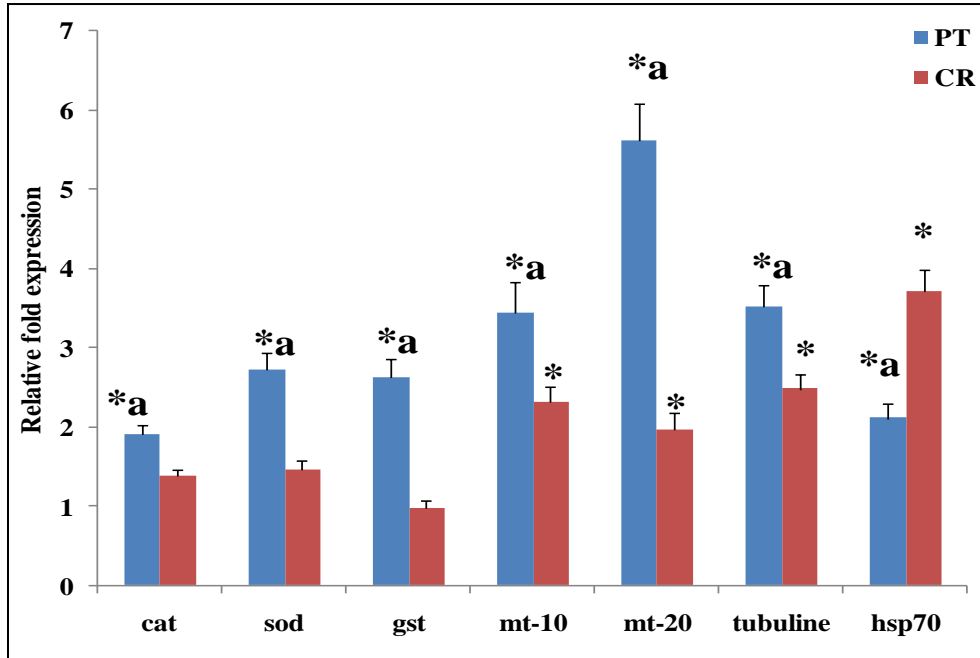


Figure 6

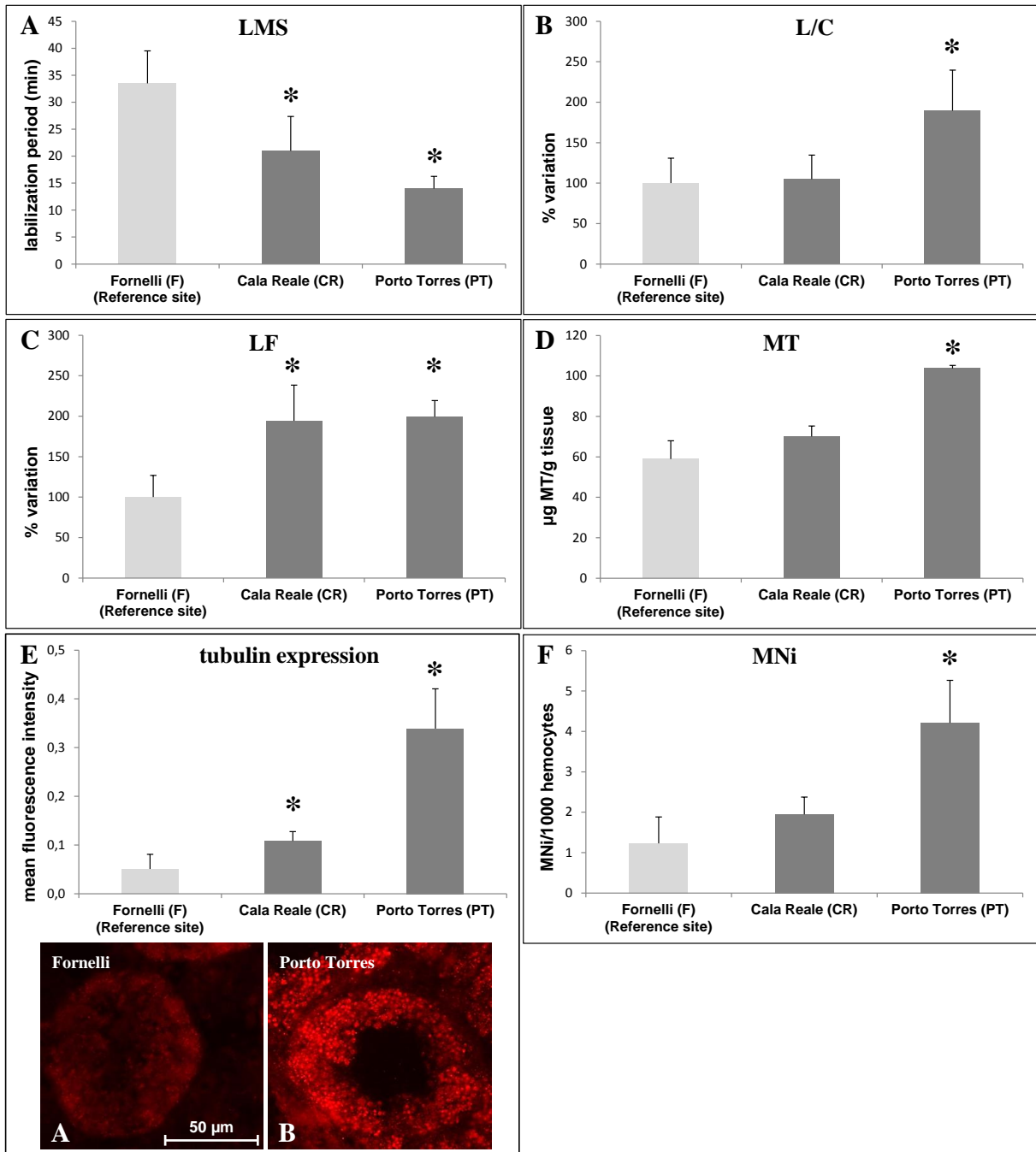


Figure 7

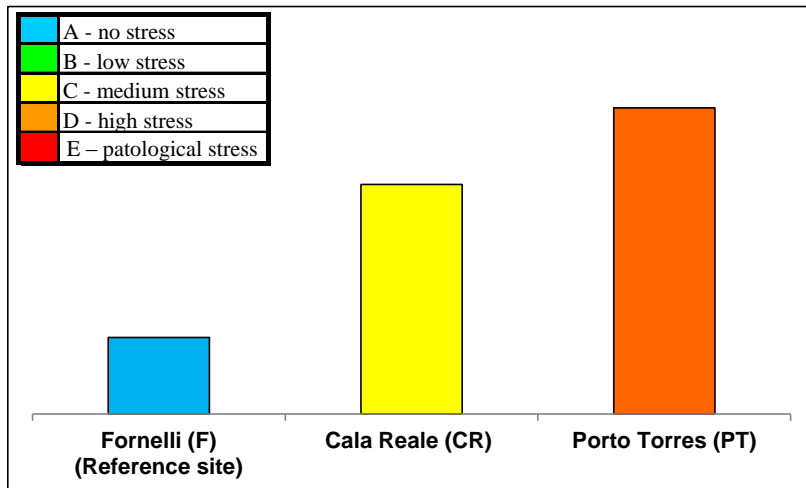


Figure 8