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Combined effects of pollution and bacterial infection in *Sabella spallanzanii* (Gmelin, 1971)

M. DARA [1,2,#], F. BERTINI [1,2,#], C. LA CORTE [1], L. BISANTI [1], M. STAROPOLI [1], J. VIZIOLI [3], D. PARRINELLO [1,2], M. CAMMARATA [1,2★], & M. G. PARISI [1,2]

[1]*Department of Earth and Marine Sciences, University of Palermo, Palermo, Italy,* [2]*NBFC, National Biodiversity Future Center, Palermo, Italy, and* [3]*U1192-Protéomique Réponse Inflammatoire Spectrométrie de Masse—PRISM, Inserm, Université de Lille, Inserm, Lille, France*

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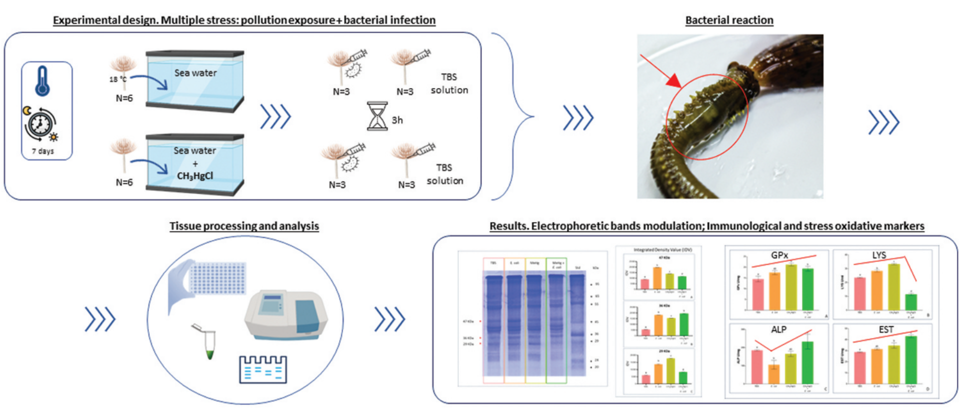
Abstract

Sabella spallanzanii is a Mediterranean tube-dwelling polychaete living in shallow water. Here we evaluate its ability to respond to individual and combined treatments of methylmercury exposure and *Escherichia coli* challenge – two typical stressors of the eutrophic environment populated by the animal. Methylmercury is one of the most dangerous and toxic pollutants in marine environments, capable of bioaccumulating and being biomagnified within the food web with consequences for marine organisms. Here, the enzymatic and immune responses of *Sabella spallanzanii* have been investigated after exposure to methylmercury, along with the combined impacts of *E. coli* infection. Fan worms were subjected to four different treatments: control conditions (buffer injection); *E. coli* injection; CH_3HgCl exposure combined, or not, with *E. coli* injection. Electrophoresis was performed to highlight possible alterations in protein patterns, differentially expressed upon bacterial and pollution challenges. Furthermore, enzymatic activities related to immune and physiological responses were assessed in whole body extracts. Our findings reveal the impact of methylmercury on the immunomodulation of *S. spallanzanii*, particularly in relation to oxidative stress and inflammatory markers. Each treatment led to a modulation of all the tested enzymatic activities, and the combined responses drastically reduced lysozyme activity.



Keywords: *Marine zoology, multiple stress, polychaetes, antioxidants, pollution, infection, immune response*

★Correspondence: M. Cammarata, Department of Earth and Marine Sciences, University of Palermo, Viale delle scienze, Edificio 16, 90128 Palermo, Italy. Email: matteo.cammarata@unipa.it
#Equal contribution.

1. Introduction

The polychaete *Sabella spallanzanii* (Gmelin, 1791) is a large and benthonic Mediterranean fan worm, generally observed in shallow and sheltered environments up to 30 m deep. It inhabits a typical self-built tube and is characterized by its large spiral feeding fan that reaches up to 10–15 cm in diameter. The fan, called the branchial crown, is usually protruded out from the tubes for sieving the water column and filtering the particulate organic matter and microorganisms it feeds on. It is considered an ecosystem engineer, able to form dense canopies of tubes extending to 50 cm above the substrate (Atalah et al. 2019). Indeed, its glandular epithelium secretes mucus in large amounts, and among its different functions, it is used as glue for the muddy particles that build up its house-tube. As in many invertebrates, mucus production also constitutes an important element determining the ability of many polychaete species to survive in their habitat (Stabili et al. 2011; Cammarata et al. 2019; Dara et al. 2022). Reproduction in the Mediterranean occurs in winter, and the lecitotrophic larvae spend more than 15 days in the pelagic phase, having a relatively high potential for dispersion (Giangrande et al. 2000). Its vernacular name, the Mediterranean fanworm, is indicative of its distribution along the Mediterranean coast, and it is commonly found in sheltered shallow areas with eutrophic conditions, such as harbors. In the last few decades, anthropogenic activities have strongly compromised the integrity of marine ecosystems due to pollution and the significant increase of many chemical pollutants, such as toxic metals and metalloids (Sumpter et al. 1996).

Sabella spallanzanii inhabits eutrophic environments, like port areas, where it can be subjected to different kind of pollutants, deriving from human industrial activities and from sewage discharges, which can cause higher concentrations of chemicals and coliform in the water basin. Therefore, this species can be considered a model to study the effects of multiple stressors on living organisms. Marine organisms, benthic animals in particular, can be potentially exposed to toxic metal pollution both in their dissolved phase and in sediments (Wang & Fisher 1999). Even at low concentrations, these pollutants can induce genetic, biochemical, physiological, and morphological changes (Zheng et al. 2019). Methylmercury (MeHg) can adversely affect organism health via toxic effects that unbalance homeostasis and immune functions. In particular, it blocks enzyme binding sites and interferes with protein synthesis, as well as the incorporation of thymidine into DNA (Bastidas & García 2004; Parisi et al. 2021c; Parrinello et al. 2017). This could impact the survival ability of several marine species, reducing their resistance and resilience to environmental stressors. Alterations of immune responses are the first indicators of the failure of an organism's defenses, with consequent direct effects on its fitness (Parisi et al. 2017c). In regular environmental conditions, disturbance factors (biological, physical, or chemical stressors) can occur simultaneously, mutually influencing one another. Indeed, they can exhibit synergistic effects (resulting in greater negative impacts than the sum of individual factors), antagonistic effects (yielding less impact than the combined sum of single components), or, infrequently, no effect on an organism's health (Michalak & Chojnacka 2014; Bellante et al. 2016; Cammarata et al. 2019; Parisi et al. 2021a; Parisi et al. 2021a; Dara et al. 2022; La Corte et al. 2023). The ability of *S. spallanzanii* to bioaccumulate xenobiotics is demonstrated by Bellante et al. (2016) who showed high Hg concentrations in tube samples from this animal (~0.811 mg/kg dry mass). Further works on *S. spallanzanii* showed its ability to bioaccumulate arsenic as an anti-predatory strategy, without compromising its biological functions (Dara et al. 2022).

Contamination of aquatic ecosystems with toxic metals (Cr, Ni, Cu, Zn, Cd, Pb, Hg, As) is a major public health issue because of their persistent accumulation in the environment (Ali et al. 2019). Human actions such as the combustion of fossil fuels, discharge of household waste and runoff from urban areas significantly contribute to the introduction of pollutants into marine ecosystems, resulting in elevated concentrations of contaminants in estuaries and coastal zones, especially those near large urban and industrial centers (Bellante et al. 2016). The toxicity of toxic metals is linked to various action mechanisms leading to adverse effects such as neurotoxicity, hepatotoxicity, and nephrotoxicity (Stohs & Bagchi 1995). The primary targets are proteins and enzymes, although toxic metals may also interact with phospholipids and DNA (Juknys et al. 2012). Among toxic metals, mercury (Hg) is regarded as a priority hazardous substance (Reg. EC 2455/2001, ATSDR, 2015; https://www.atsdr.cdc.gov/spl/accessed on 13 December 2023), playing an important role in the anthropogenic pollution of aquatic ecosystems (Driscoll & McElroy 1997; Nuran Ercal et al. 2001; Corbitt et al. 2011; Cappello et al. 2016). It has been clearly established that, due to its ability to bioaccumulate and be biomagnified through the food web, its high toxicity can affect living organisms at multiple levels (Cappello et al. 2016).

The bioaccumulation of mercury in marine organisms, including those used for food consumption, suggests a risk for biomagnification in humans. Alho and Vieira (1997) evaluated the contamination and biomagnification of mercury and other pollutants at different levels, measuring contamination in sediments, filter-feeder mollusks (clams), and vertebrates (fish and birds). They found higher mercury levels in the tissues of piscivorous birds than clam feeders, confirming the biomagnification of mercury and its higher content in organisms of higher trophic levels and encouraging more attention to its unhealthy effects on humans through seafood, in particular fish and tuna consumption (Alho & Vieira 1997; Médieu et al. 2023; *Minamata Convention on Mercury*, 2023; Reg. EC 1881, 2006).

In coastal waters, reactive forms of Hg are present at higher concentrations (Gworek et al. 2016) compared to the open sea, where Hg concentrations are estimated to be in the range of 0.5–3.0 ng/L (Faganeli et al. 2012), and it can be found in both organic and inorganic forms. Mercury pollution and its toxicity in aquatic systems is mainly due to its methylation in sediments to organic methylmercury (MeHg), which is the most toxic and bioaccumulating form of Hg in marine species (Sizmur et al. 2013). It is important to investigate its biological effects on living organisms since this factor may be responsible for alterations in immune functions of animals through the accumulation of toxic metals (Sánchez Uría & Sanz-Medel 1998; Ikingura & Akagi 1999; Wang & Fisher 1999; Allen & Moore 2004; Bastidas & García 2004; Ipolyi et al. 2004; Leermakers et al. 2005; Parisi et al. 2017a; Connon et al. 2012; Chiarelli & Roccheri 2014; Hamza-Chaffai 2014; Parrinello et al. 2017; Parisi et al. 2019; Parmar et al. 2016; Zheng et al. 2019; La Corte et al. 2023). During the first responses of invertebrates to chemical agents or environmental stressors, enzymatic activity plays a fundamental role as a diagnostic tool for natural contamination screening, and it is frequently used as a biochemical stress marker (Mukherjee et al. 2021; Lusic et al. 2022). Here, we explored glutathione peroxidase (GPx), lysozyme (LYS), esterase (EST) and alkaline phosphatase (ALP) enzyme activities. They can be considered highly effective indicators, playing a fundamental role in adaptation to extreme environments and revealing the organism's responses to stressful conditions. Indeed, these enzymes play key roles in processes associated with cellular immunity and phagocytosis.

Innate immune responses generally produce cytotoxic radicals, such as reactive oxygen species (ROS), which can lead to oxidative stress and cause tissue damage (Gaete et al. 2017). Antioxidants (such as peroxidase), which readily scavenge oxygen radicals, are critical to preventing self-damage (Cerenius et al. 2010) and are often abundant in areas of tissue injury and pathogen infection (Halliwell & Gutteridge 2015). LYS functions as a bactericidal enzyme, breaking down the β-1,4 glycosidic bonds within peptidoglycan, the main component of both Gram-negative and Gram-positive bacteria cell walls (Li et al. 2008). Additionally, in annelids, the presence of LYS has been detected in mucus, in various tissue types, and in the coelomic fluid (Marcano et al. 1997; Stabili et al. 2014, 2019). ALP and EST enzymes contribute to a deeper comprehension of how animals adapt and maintain energetic balance under stressful conditions. These enzymes, involved in synthesis, hydrolysis reactions and catabolic pathways, play a role in different metabolic functions including digestive, inflammatory and detoxification processes (Guardiola et al. 2015; Parisi et al. 2017b).

The aim of this study was to elucidate the effect on *S. spallanzanii* immune responsiveness after individual and combined treatments of MeHg exposure and bacterial challenge.

2. Materials and methods

2.1. Polychaete collection and experimental design

The sampling of adult specimens of *S. spallanzanii* was carried out during the spring in a port area (Cala di Palermo, Italy). After being cleaned of epiphytes, individuals were kept for acclimatization in tanks filled with oxygenated filtered seawater at 18 ±1°C for a period of 7 days, before proceeding with the experiments, which were all performed at the same temperature.

The specimens were divided into two groups: six were maintained in filtered sea water serving as the control exposure treatment and six were exposed to methylmercury (CH_3HgCl) dissolved in filtered sea water (final concentration 10^{-6} M) (Wang & Fisher 1999; Parrinello et al. 2017) under the same conditions of oxygenated sea water at 18 ±1°C, for 4 days. No animals died during the experiments. Viability was verified by checking the active reaction of the branchial crown.

Then, three specimens from each tank were inoculated with Tris-Buffered Saline (TBS) buffer (150 mm NaCl, 10 mm Tris-HCl, pH 7.4), serving as control treatment specimens for the inoculation, while the others were injected with 1×10^7 bacteria *Escherichia coli* (ATCC 25,922) (Chrisope Technologies, Louisiana, USA) suspended in TBS

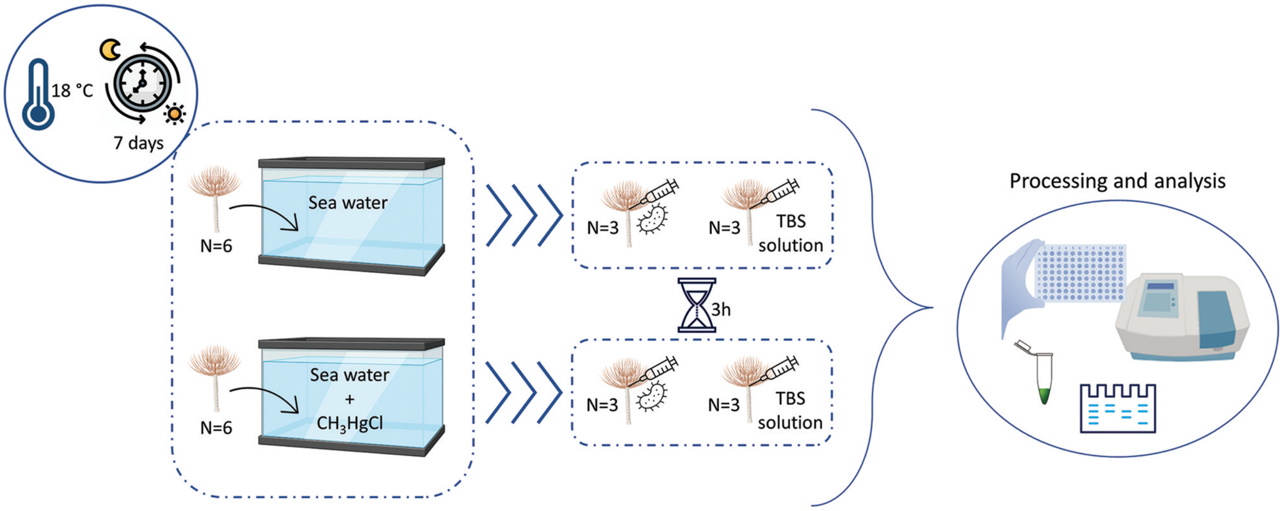


Figure 1. Illustration of the experimental design.

(see below, section 2.2) (Figure 1). The animals were gently removed from their tube, and injections of 100 µL of heat-killed *E. coli* and TBS were made in the coelom, in the upper part of the abdomen, just under the thorax to avoid inducing fan autotomy. Three hours later, the specimens were collected for subsequent analysis. Experiments and analyses were done in triplicate. Chemicals and reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. *Bacterial suspensions*

Escherichia coli bacteria were grown in tryptic soy broth at 25°C in a Gallenkamp incubator under continuous shaking (120 rpm) until the bacterial stationary phase. Heat-killed bacteria (120°C, 1 atm for 20 min) were centrifuged at 6000 × *g* for 15 min at 4°C, washed in sterile PBS and resuspended to achieve 1×10^9 cells/mL. To standardize the number of cells to use in the experiments, we used the correlation between the number of cells assessed by plate count and their absorbance at 600 nm in culture. Heat-killed bacteria were kept at 4°C until used in the bacterial inoculation.

2.3. *Protein extraction*

Tissues from the whole body of *S. spallanzanii* were homogenized on ice using a homogenizer (Omni TH, TH-02) in TBS solution and centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was gathered and preserved at −20°C until the analyses.

2.4. *Protein concentration*

Protein concentration (mg/mL) in tissue extract supernatant was measured using the Bradford method (1976), reading absorbance at 595 nm in a RAYTO RT-2100C spectrometer (Rayto Life and Analytical Sciences Co., Ltd, Shenzen, P. R. China). TBS solution was used as a blank, and a calibration curve was generated using serial dilutions of bovine serum albumin (BSA) (Bradford 1976). Prior to conducting enzymatic assays, extracts were standardized to 0.5 mg/mL.

2.5. *SDS-PAGE*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%) was performed to separate proteins from *S. spallanzanii* body extracts according to the procedure described by (Dara et al. 2021). The gel was calibrated with molecular mass markers (S8445 – Sigma-Aldrich, USA). The electrophoretic run started with 10 min at 60 V, and then proceeded for 1 h at 180 V in Tris-Glycine (Trizma Base 25 mm, Glycine 192 mm, 1% w/v SDS, pH 8.3) buffer under non-denaturing conditions. The gel was stained with Coomassie brilliant blue and bleached with 10% acetic acid and 40% methanol in distilled water. The reaction was stopped with distilled water. The intensity of the main bands was quantified by densitometry (integrated density value – IDV) using the Image J software package (http://rsbweb.nih.gov/ij/download.html).

2.6. *Enzymatic activities*

GPx activity was assessed in triplicate in a 96-well flat-bottomed plate, using 50 µL of sample and 100 µL of TMB (3.3”, 5.5”−tetramethylbenzidine). The plate was then incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 50 µL of 2 M sulfuric acid (H_2SO_4). The absorbance (Abs), representing the produced GPx, was measured at 450 nm using a microplate reader (RAYTO RT-2100C) and was quantified in U/mg of protein using the equation U/mg = Abs * Vf/CP, where Vf is the final volume of the well, and CP is the protein concentration of the sample. TBS was used as blank.

To evaluate LYS activity in *S. spallanzanii* protein extracts, a turbidimetric assay modified for a microplate reader was used (Sutton et al. 2006). First, 15 µL of each sample was added in a 96-well flat-bottomed plate, followed by incubation with 250 µL of *Micrococcus lysodeicticus* (ATCC 4698) suspension (absorbance at 405 nm = 0.5–0.7). Assays were performed in triplicate, and 15 µL of TBS solution was used for the control. The turbidity difference of *Micrococcus lysodeikticus suspension*, measuring the absorbance (at 450 nm) before and after the incubation of samples (20 min at 37°C), was used to measure the LYS activity. The definition of a LYS unit was based on the sample amount causing a reduction in absorbance by 0.001/min (U min^{-1}), and U/mL was determined using the formula U/mL = (Δ abs/min^{-1} * dilution factor * 1000)/enzyme volume in buffer (Sutton et al. 2006).

ALP activity was estimated by incubating 50 µL protein extract samples with the same volume of the substrate 4 mm p-nitrophenyl phosphate diluted in 100 mm ammonium bicarbonate and 1 mm $MgCl_2$ (pH 7.8). The enzymatic kinetics were assessed following the method outlined by Parisi et al. (2021a, 2021b). Briefly, absorbance was measured at 405 nm at regular intervals, ranging from 5 min to 1 h at 405 nm using a microplate reader, as described above. A single unit (U) of activity was defined as the quantity of enzyme necessary to liberate 1 µmol of p-nitrophenol in 1 min.

EST activity was estimated following the method described by La Corte et al. (2023): an equal volume of sample was mixed with 0.4 mm p-nitrophenyl myristate substrate +100 mm ammonium bicarbonate with 0.5% of Triton X-100 (pH 7.8). The assessment of increased optical density and activity were conducted in a manner like that used for ALP.

2.7. *Statistical analysis*

Enzymatic assay data analyses and processing were performed by one-way analysis of variance (ANOVA), utilizing GraphPad Prism Version 8.0.0. to evaluate the interaction and possible significance between the treated samples and the control. The post-hoc test used was the Tukey test. The differences in data were considered statistically significant for * $p < .05$; ** $p < .01$ and *** $p < .001$.

3. Results

3.1. *Macroscopic remarks*

No deaths occurred during the 4-day experimental period. All specimens appeared to be free of any evident morphological or color alterations of the body, and there was no fanning autotomy in any treatment. After bacterial inoculation, a yellowish reaction zone formed in the injection area (Figure 2) 3 h after the treatment, in line with the macroscopic observations reported for inoculated animals by La Corte et al. (2023).

3.2. *GPx activity*

GPx activity, assessed in *S. spallanzanii* on entire protein extracts, was higher in samples exposed to the different treatments compared to controls (TBS injected). A statistically significant increase in enzymatic activity was observed for CH_3HgCl and the combined treatment, reaching values over 20 U/mg

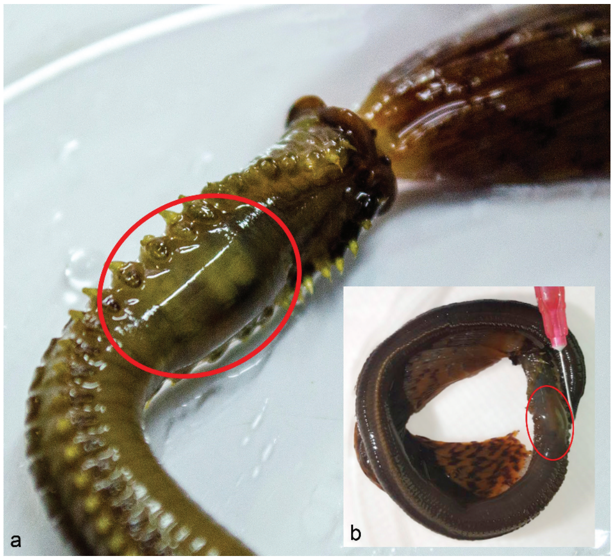


Figure 2. Reaction observed around bacterial inoculation (circled in red in (a) and (b)) 3 h after injection.

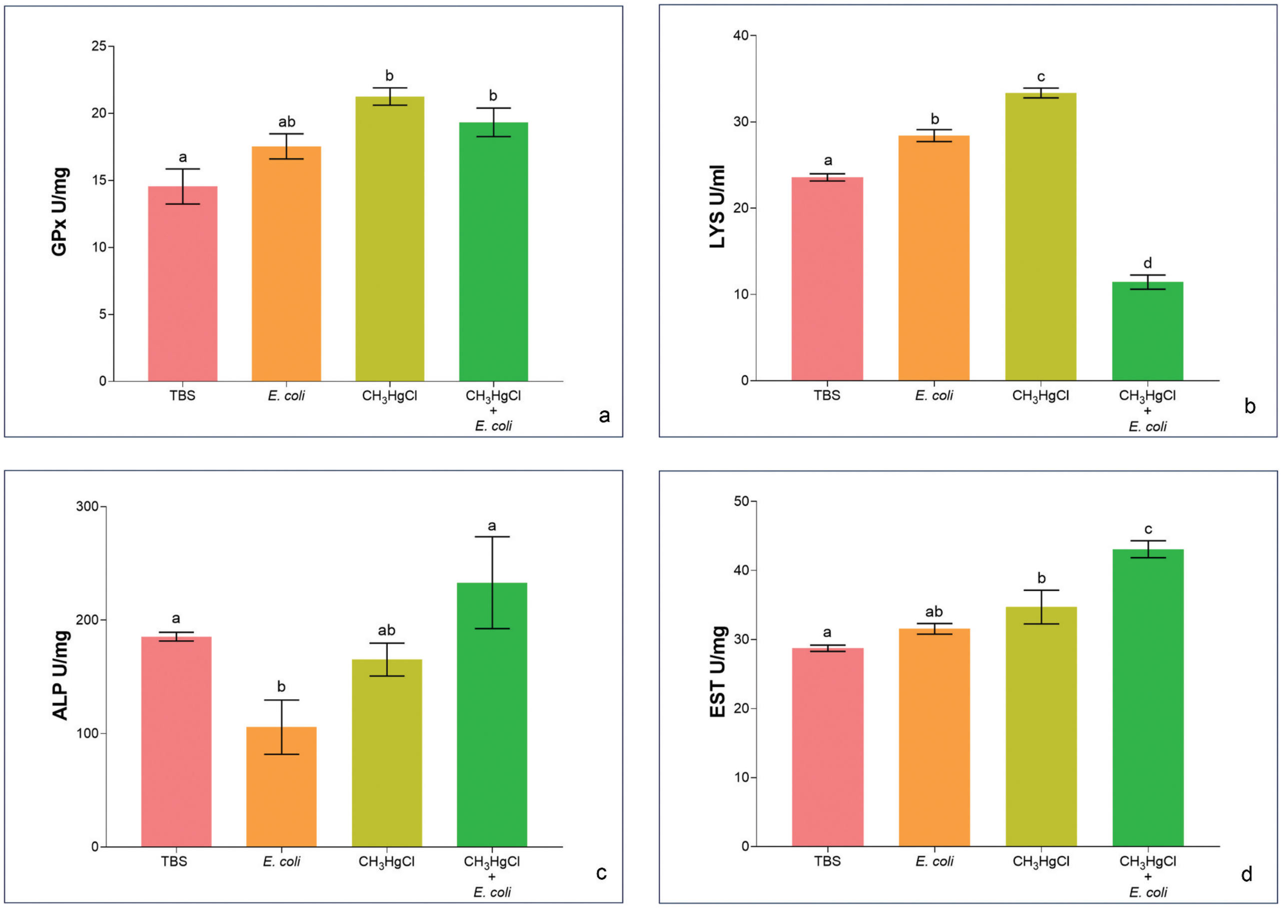


Figure 3. Graphs depicting the enzymatic responses of glutathione peroxidase (GPx) (a), lysozyme (LYS) (b), alkaline phosphatase (ALP) (c), and esterase (EST) (d) in whole-protein extracts of *S. spallanzanii*. The data underwent testing for ANOVA; assumptions and differences among groups were illustrated through one-way ANOVA as mean ± standard error of the mean. Lowercase letters denote statistically significant differences between treatments. Significance was considered for differences between means at $p < 0.05$.

following exposure to the pollutant (Figure 3(a)). The multiple comparison Tukey test showed a p value <.05 between the control group and that exposed to methylmercury ($p = .001$), and between the control group and the group with combined treatment ($p = .02$).

3.3. LYS activity

The enzymatic activity of samples showed a statistically significant increase in specimens treated with the *E. coli* injection and in those exposed to CH_3HgCl with respect to the control (TBS) (Figure 3(b)). In contrast, a significant decrease in LYS activity occurred following the combined treatment with *E. coli* + pollutant. The Tukey test highlighted significant differences between organisms injected with *E. coli* and CH_3HgCl + *E. coli* ($p < .0001$), and between samples subjected only to CH_3HgCl vs CH_3HgCl + *E. coli* ($p < .0001$).

3.4. ALP activity

Overall, the ALP activity of extracts was significantly inhibited by the *E. coli* injection compared to the control condition and the combined treatment (Figure 3(c)). The Tukey post-hoc test showed significant differences between the *E. coli* group and the control group ($p = .0446$).

3.5. EST activity

All treatments caused a significant increase of EST activity in body extracts compared with the control group (TBS) (Figure 3(d)). The Tukey post-hoc test evidenced a $p < .0001$ difference between the combined treatment vs control group, and vs *E. coli* injection. The exposure to methylmercury showed a p value $< .05$ vs the control group and the combined treatment. Again, one-way ANOVA revealed generally significant differences between the experimental treatments ($p < .0001$).

3.6. SDS-PAGE and integrated density analyses

The electrophoretic analysis revealed evident protein bands similar among the treatment lines (Figure 4). In particular, the effect caused by each treatment was evidenced by the modulation of certain bands (47, 36, 29 kDa) (Figure 3). The IDVs measured for these modulated bands were generally higher in treatments than in controls (TBS) (Figure 5). For the 47 kDa band (Figure 5(a)), the highest IDV was observed for the bacterial injection, whereas it appeared to be significantly reduced for the exposure to MeHg and the combined treatments. The IDVs of the 36 kDa bands revealed a similar increase upon bacterial infection, independently from the presence of the pollutant, which also induced a significant rise in values compared to the control (Figure 5(b)). MeHg exposure greatly affected the amount of the 29 kDa band (Figure 5(c)), which was also positively modulated by bacterial infection. As observed for the 47kDa band, the combined treatment was the least effective on the 29 kDa band, inducing the smallest increase compared to the control.

4. Discussion

Since the Minamata Bay poisoning case in the 1960s (Harada 1995), concerns about Hg pollution and its impact on the marine environment have been the driving force behind extensive research activity on this phenomenon (Bickham et al. 2000). It is possible to study toxic metals and their potential toxicity in aquatic environments and on tissues of specific organisms possessing the specific characteristics of bioindicators, which can therefore be used as biomonitoring tools for the marine-coastal environment.

Hg is mainly accumulated in the sediment, and *S. spallanzanii*, as a filter feeder, is in close contact with resuspended sediment particles that it uses to build its tube, making it potentially more vulnerable compared to organisms with predatory behavior (Brandão et al. 2015). Our results on polychaetes exposed to MeHg have shown an immunological response to pollutant exposure. This is consistent with observations of the effects on other benthic invertebrates such as sea anemones, ascidians, and mussels (Cammarata et al. 2007; Parisi et al. 2021c; Parrinello et al. 2017). Indeed, metals like

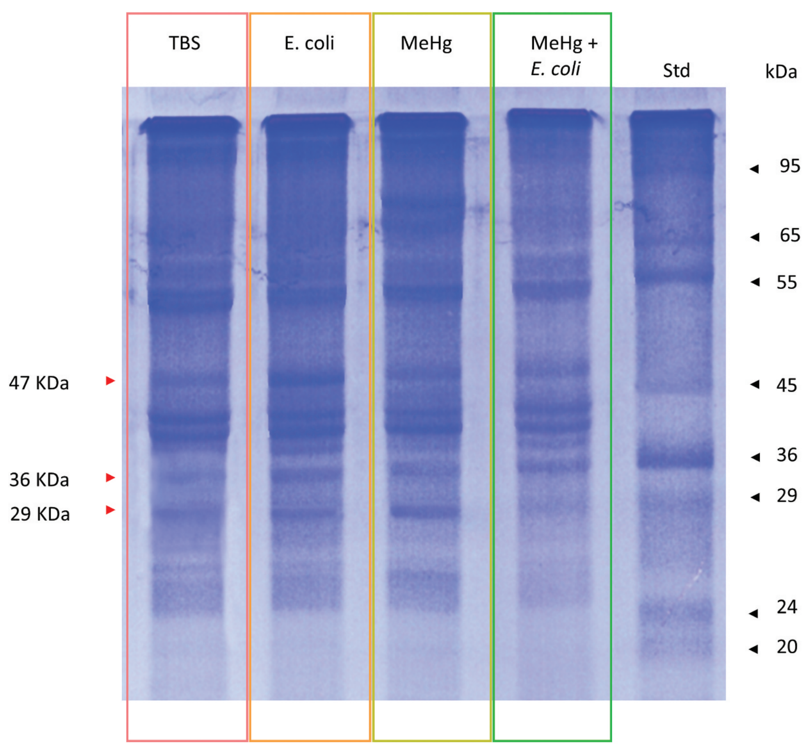
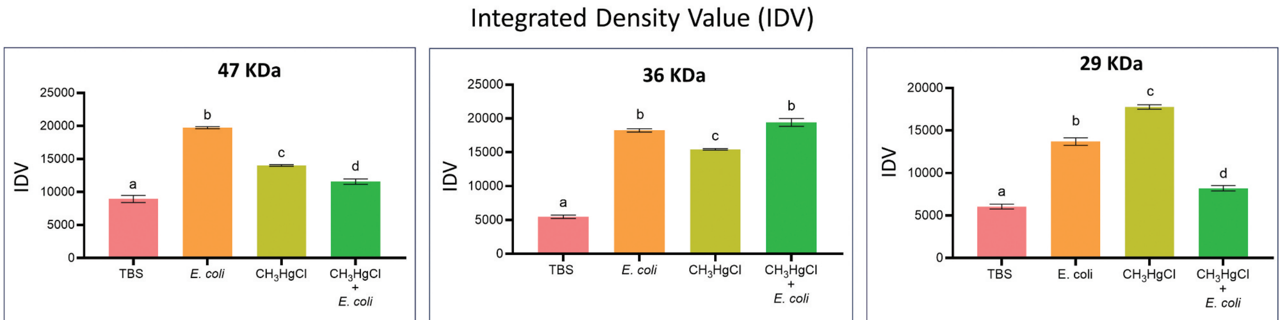


Figure 4. Example of SDS-PAGE (10%) under non-reducing conditions of *S. spallanzanii* extracts: TBS; *E. coli*; CH_3HgCl; *E. coli* + CH_3HgCl. The molecular masses (kDa) of markers are displayed on the left (Std). Red arrow heads indicate the protein bands, showing the main variations in intensity in the different samples. Black arrow heads indicate the molecular mass of the standard protein bands.



	Treatments	IDV Mean value	Standard error
47 kDa	TBS	8939,3	311,953
	E. coli	19735	92,828
	MeHg	14009,7	73,976
	MeHg + E. coli	11562,3	234,806

a

	Treatments	IDV Mean value	Standard error
36 kDa	TBS	5492,3	251,306
	E. coli	18245,3	246,889
	MeHg	15432,6	112,754
	MeHg + E. coli	19410,7	575,041

b

	Treatments	IDV Mean value	Standard error
29 kDa	TBS	6052	294,617
	E. Coli	13700,7	444,436
	MeHg	17763	254,215
	MeHg + E. coli	8208,3	321,738

c

Figure 5. Histograms representing the integrated density values (IDVs) of the bands differently evidenced in the SDS-PAGE analyses in the control and experimental treatments. (a, b, c) Histograms and IDV tables corresponding to the 47, 36 and 29 kDa bands, respectively. Lowercase letters refer to statistically significant differences between treatments. Significance was attributed to differences between means at $p < .05$. The table beneath each graph presents a summary of the enzyme results from the ANOVA.

methylmercury can affect organisms' homeostasis, provoking modifications in immune responses and signaling pathways which could compromise the performance and fitness of marine species (Cammarata et al. 2007; Parisi et al. 2021c; Parrinello et al. 2017; Zheng et al. 2019). This study investigated the immune responses of *S. spallanzanii*, considering the entire body of the organisms. Fan worms were exposed separately and simultaneously to chemical and biotic stress to evaluate the effects on immune response using validated biomarkers. Indeed, the multi-biomarker approach can be useful for identifying which constituents of complex pollution scenarios are driving physiological responses (Díaz-Jaramillo et al. 2011).

Most toxicological studies done on annelids focus on their physiological and biochemical responses and seldom depict morphological modification of the body following pollutant exposure. The delicate removal of the animals from their tube made it possible to evaluate any effects deriving from the various treatments on the body of the polychaetes. A recent study showed a local reaction after bacterial injection and exposure to copper sulfate (La Corte et al. 2023). In our case, TBS-injected worms did not reveal any swelling or morphological alterations at the inoculation site. Only the specimens injected with *E. coli* showed a visible reaction, with an evident yellowish bloated area at the injection site. This is a clear sign of a rapid antimicrobial response to the bacterial materials inoculated. LPS and heat-killed bacteria, even if not living cells, have been widely used as strong elements to trigger the immunological response of marine organisms to study their immune defensive mechanisms (Bisanti et al. 2024, 2024; Conforto et al. 2021; Parrinello et al., 2017; Parisi et al., 2015, 2019, 2022). Indeed, lipopolysaccharide (LPS), constituent of Gram-negative bacteria, can trigger immune responses via the toll-like receptor 4 (TLR4) even isolated or in killed bacteria (Bisanti et al. 2023; Bisanti et al. 2024a, 2024b; Conforto et al. 2021; Nicolò et al. 2016; Parisi et al. 2022, 2019, 2015). When the tissues are damaged by bacteria or injury, swelling is generated to isolate the pathogens and confine the infection (Trapani et al. 2016; La Corte et al. 2023).

In this work, all the treatments induced the modulation of the analyzed enzymes, compared to control specimens, in particular in the combined treatment which showed synergistic or antagonistic effects causing an increase or decrease in activities. Indeed, previous work on animal filter models has shown that exposure to mercury can lead to cellular changes at the cytoskeletal and pseudopod level, and to a consequent increase in cellular mortality (Parrinello et al. 2017; Parisi et al. 2021), opening a new possible observation scenario of multi-stress phenomena. We believe that the data collected here allow us to validate the role of *S. spallanzanii* as a bioindicator for the aquatic environment, and the use of its immune and physiological responses as biomarkers is suitable for the assessment of exposure to pollutants.

It is known that environmental stressors due to chemical agent exposures cause oxidative stress in marine animals, stimulating the production of ROS in the subcellular organelles, which are dangerous to the organism at high concentrations (Gaete et al. 2017). Oxidative stress responses include the antioxidant enzyme GPx, and its activity is recognized as arising in response to environmental pollutants (Almeida et al. 2002; Sayeed et al. 2003; Zhang et al. 2004). Our data show that GPx activity was significantly stimulated following the treatments, compared to the control group, in accordance with Tian et al. (2014) and their studies on another polychaete species, *Perinereis aibuhitensis*, in which activation by lead exposure (another highly toxic metal) revealed the greatly disruptive capacity of this pollutant for glutathione-dependent enzymes. Hg induces oxidative stress by H_2O_2 production, whereas GPxs are crucial for peroxide removal. Glutathione contains sulfhydryl group (SH-groups) that play a central role in intracellular Hg sequestration as the main thiol pool (Colacevich et al. 2011). Therefore, GPx appears to play a significant role in the reduction of mercury-induced oxidative stress. Increases in hepatic GPx activity were also observed in fishes like the Atlantic salmon (*Salmo salar*) and the black bullhead (*Ictalurus melas*) when exposed to $HgCl_2$ (Berntssen et al. 2003; Elia et al. 2003).

Another factor that participates in protection against pathogenic agents is LYS. The constitutive values of this bacteriolytic enzyme observed in control specimens could reflect the presence of a basal, functional mechanism that protects the organism from bacteria living in its environment and controls their natural microbiome (Marcano et al. 1997; Cuvillier-Hot et al. 2014). Our data show that LYS enzymatic activity increases following the bacterial inoculum, in line with Marcano et al. (1997) who reported that the naturally occurring LYS activity in the coelomic fluid of polychaetas can be induced by bacterial injection. The highest activity was registered for specimens exposed to methylmercury alone. Previous studies, carried out with other invertebrate species, showed an increase in LYS mRNA expression after toxic metal exposure for more than 3 days, indicating the occurrence of non-specific immune responses caused by long-term exposure (Fang et al. 2013). In contrast to the results reported by Gagnaire et al. (2007), where LYS was up-regulated in *Crassostrea gigas* following simultaneous exposure to pesticides and bacteria, the combined treatment (MeHg + *E. coli*) in our study led to the inhibition of this enzyme (Gagnaire et al. 2007). Fang et al. (2013), however, demonstrated that LYS expression can potentially decrease on day 5 and be inhibited on day 7 after bacterial injection and exposure to mercury and cadmium. The antibacterial activity of coelomic fluid, associated with LYS-like substances and inducible humoral molecules, supports coelomocyte reactions in the annelid defense system. Therefore, an inhibition of this enzyme following the combined treatment is probably due to the exposure to MeHg, which can potentially alter cellular functions, modifying the immune response of the organisms (Fang et al. 2013). Reduced ALP activity may be related to the oxidative damage induced by MeHg, suggesting that enzyme inhibition is a crucial mechanism through which mercury elicits harmful effects (Gaete et al. 2017). The decline in phosphatase activity may indicate potential cellular dysfunction and compromised immunity, as indicated by (Gautam et al. 2020) in a study involving earthworms exposed to a metal-polluted soil.

Finally, the data presented in this study reveal an increase in EST activity across the different treatments, with a notable peak observed in the combined challenge compared to the control group. This surge in activity could be attributed to the oxidative stress induced by methylmercury, exacerbated by the introduction of *E. coli*. Different studies, further, provide evidence that EST activity may be influenced by a wide range of contaminants, including toxic metals (Guilhermino et al. 1998; Cajaraville et al. 2000; Bonacci et al. 2004).

The different treatments' effects on *S. spallanzanii* also resulted in different protein patterns, showed by the electrophoretic analyses, indicating specific modulation in responses to pathogens after chemical conditioning. Bacteria inoculum stimulated the production of proteins with molecular masses around 47, 36, and 29 kDa. A significant IDV decrease was observed for bands with molecular mass around 47 and 29 kDa following exposure to MeHg and the combined treatment. In contrast, the combined treatment caused the IDV of the 36 KDa band to increase. Considering the nature of the stimulation to which the specimens were exposed by the treatment, the modulated bands could be attributed to proteins linked to host reaction and to immune response modulation (La Corte et al. 2023). Indeed, the predicted molecular masses are consistent with those of proteins present in annelids typical of the antibacterial response, such as for lysenin and fetidin, proteins characterized by hemolytic activity and antibacterial properties (Lassegues et al. 1997; Milochau et al. 1997; Yamaji et al.

1998; Cooper et al. 2001; Kiyokawa et al. 2004; Bruhn et al. 2006; Schenk & Hoeger 2020). Fetidin, existing as two isoforms with molecular masses of 40 and 45 kDa, is released by coelomocytes and represents 20% of coelomic fluid proteins in *Eisenia fetida*; the two isoforms represent different glycosylation states of a 34 kDa peptide, which contains a peroxidase domain (Valembois et al. 1982; Vaillier et al. 1985; Lassegues et al. 1997; Milochau et al. 1997; Schenk & Hoeger 2020). Lysenin (41 kDa) is synthesized and released into the coelomic fluid of the earthworm *E. fetida* by coelomocytes (Ohta et al. 2000; Opper et al. 2013) and acts as a pore-forming protein causing cytolysis (Opper et al. 2013; Bokori-Brown et al. 2016). Another protein found in the coelomic fluid of *Eisenia*, known as eiseniapore (38 kDa), exhibits cytolytic activity (Lange et al. 1999). A further 42 kDa coelomocyte cytolytic factor 1 (CCF-1), first extracted from *E. fetida* (Bilej et al. 1995), has been identified in various earthworm species belonging to the genera *Aporrectodea* and *Lumbricus*, as well as the species *Dendrobaena veneta* (Šilerová et al. 2006). In the polychaete *Nereis virens*, a fibrinolytic serine-proteinase (29 kDa) has been detected in the coelomic fluid (Zhang et al. 2007). These proteinases are synthesized and stored within coelomocytes. They are subsequently released to confront immune challenges, triggering defensive reactions (Roch et al. 1998).

Data presented here were obtained analyzing *S. spallanzanii* total body protein extracts. Further studies are necessary to characterize the proteins modulated in the whole-body extracts from *S. spallanzanii* and establish their possible correspondence with other known annelid cytolytic factors, and will elucidate the bio-marker modulation related to antioxidant defenses in cell- and tissue-specific organs (e.g. the fan, the coelomic fluid, or the coelomic cell type).

5. Conclusion

The data reported here contribute to elucidating the effects of methylmercury pollution in aquatic habitats and their biological effects on the immune functioning of animals with accumulated toxic metals.

The various alterations in the expression of these proteins under metal exposures and the influence of the enzymatic activity here presented may result in immunosuppression, creating concrete risks for the species and contributing to the loss of population biodiversity and alteration of ecosystem equilibrium. Indeed, the use of sentinel species as bioindicators can reveal anticipatory signals in monitoring organisms and their environment. Pollutants might cause toxic effects at different scales: starting at the molecular level, initiating a cascade of responses, their action can be effective on higher levels, ranging from tissue damage to organism health, up to the population level and, finally, evolutionary pressure on the entire species, including extinction. As demonstrated here, the effect of the pollutants could interfere with the immunological defense of the organisms, contributing to the microbiological risks to which organisms are subjected.

Therefore, it is imperative to reduce the use of mercury due to the severe risks it poses for aquatic biota, biodiversity, and the health of human populations that rely on fish as their primary source of protein. We confirmed that *S. spallanzanii* is a useful and reliable bioindicator in both ecotoxicological and immunological studies. We showed a modulation of immunoreactivity that could affect organism health, altering homeostasis.

In conclusion, our results demonstrate that polychaetes subjected to combined treatment presented different patterns of enzymatic responses. Bacterial infections may produce different toxic scenarios in the presence of methylmercury, confirming the possible interactions between stressors (biological and pollutant in our study), as previously described.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Credits

Dara, Mariano: conceptualization, data curation, formal analysis, investigation, methodology, funding acquisition, writing – original draft, writing – review and editing; **Bertini, Federica**: conceptualization, sata curation, formal analysis, investigation, methodology, writing – original draft, writing – review and editing; **La Corte, Claudia**: conceptualization, data curation, formal analysis, investigation, methodology, roles/writing – original draft, writing – review and editing; **Bisanti, Luca**: formal analysis, investigation, writing – review and editing; **Staropoli, Mariele**: formal analysis, investigation, writing – review and editing; **Vizioli, Jacopo**: formal analysis, investigation, roles/writing – original draft, writing – review and editing; **Parrinello, Daniela**: data curation, funding acquisition, roles/writing - original draft, writing – review and editing; **Cammarata, Matteo**: data curation, funding acquisition, project administration, supervision, validation, roles/writing – original draft, writing – review and editing; **Parisi, Maria Giovanna**: funding acquisition, roles/writing – original draft, writing – review and editing.

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ORCID

M. Dara http://orcid.org/0000-0002-1122-6131
F. Bertini http://orcid.org/0000-0002-8623-0725
C. La Corte http://orcid.org/0000-0002-5816-2657
L. Bisanti http://orcid.org/0000-0001-8213-3154
M. Staropoli http://orcid.org/0009-0002-4049-6744
J. Vizioli http://orcid.org/0000-0002-4310-8033
D. Parrinello http://orcid.org/0000-0001-9224-7737
M. Cammarata http://orcid.org/0000-0002-5497-1246
M. G. Parisi http://orcid.org/0000-0001-5462-0596

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