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Effects of seismic water guns on the peristomial membrane of sea urchins (*Arbacia lixula*, Linnaeus 1758)

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ABSTRACT

The seismic water gun is widely used and plays an important role in seabed imaging acquisition; however, acoustic impacts on marine organisms are currently poorly understood. The aim of this study was to analyse the biochemical responses on the peristomial membrane (PM) of the sea urchin, *Arbacia lixula*, when exposed to water gun shots in open water. The PM (located around the mouth) is involved in vital functions, such as nutrition and protection. Individuals of sea urchins (n = 7 for each time slot) were sampled before, at the end, and at intervals of 3 h and 24 h after acoustic emission (duration of 20 min). Significant increases in superoxide dismutase, peroxidase, esterase and alkaline were observed immediately after water gun shots, highlighting an increase in the oxidative and inflammatory state of the tissue. Our results showed that acoustic impacts could interfere with PM vital functions, compromising the health, survival and ultimately the conservation of the species. Understanding these effects is crucial to predicting consequences on sea urchin populations and marine ecosystems.

1. Introduction

Marine ecosystems have their own soundscape, defined as the sum of natural (animal and geophysical sounds) and anthropogenic components (sounds generated by human activities) (Pijanowski et al., 2011). Acoustic signals propagate faster and farther in water compared to other signals (i.e. faster than chemical and farther than electromagnetic) and marine species have developed different mechanisms throughout their evolution to detect and produce sound carrying vital information (Duarte et al., 2021). The anthropogenic component of the soundscape is increasing rapidly due to both intensification of human activities in the ocean and climate change, the latter of which increases acoustic energy absorption, thus favouring acoustic propagation (Halpern et al., 2008; Slabbekoorn et al., 2010; Duarte et al., 2021). Anthropogenic noise constitutes a stressful condition and is recognized as one of the main causes of pollution in the 21st century (Halperin, 2014; Duarte et al., 2021). The threat to marine ecosystems is such that noise is now included in the descriptors of the state of the marine environment in the Marine Strategy Framework Directive (2008/56/EC - Marine Strategy Framework Directive (MSFD)).

Several types of anthropogenic activities cause increases in underwater noise, including maritime traffic, drilling, construction, offshore wind farms, sonar, military exercises, airguns and water guns (Hawkins et al., 2015). These latter are widely used to provide a better understanding of deep seabed structure and they produce the highest energy-pulsed sounds. Water guns are a relatively new marine seismic sound source; they produce acoustic signals using an implosive rather than explosive mechanism, such as that used by airguns. The level of sound produced in a seismic survey varies with the number of guns used in the array (a single gun or a double-gun array towed by a ship), the size of the guns and the position of the array (bottom vs. surface), with a peak that can reach a $SL_{0\text{-peak}}$ of 253 dB re 1μPa (Mihailov, 2020). When describing air/water gun sound sources, it is useful to consider metrics which include both pressure variation and particle motion in three

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directions. These two physical quantities are related to each other (Ceraulo et al., 2016; Hazelwood and Macey, 2016), although marine species can be more sensitive to one quantity rather than another. Marine vertebrates, such as marine mammals, are sensitive to sound pressure. Fish are sensitivity to particle motion and the presence of the swim bladder and its connection with the ear increase their sensitivity to pressure (Popper et al., 2014). For example, Clupeidae that have a special structure mechanically linking the swim bladder to the ear are sensitive primarily to sound pressure (Ladich and Popper, 2004). Invertebrates, that generally do not have gas-filled bladders (Carroll et al., 2017; Solé et al., 2023), are especially sensitive to particle motion and not to pressure (Hawkins and Popper, 2017). By introducing high acoustic energy into the sea, seismic surveys alter the natural soundscape for weeks with a spatial scale of tens of kilometres (Duarte et al., 2021). Although countries often apply a precautionary approach to seismic surveys by limiting the length and duration of exploration (Lewandowski, 2015), the effect of this technique on marine life is still poorly understood. The low frequencies emitted by this technique (impulsive noise with most of the energy below 1 kHz) fall within the frequency range of sounds detected by many marine species (Popper and Fay, 2011), thus raising concerns about effects on biodiversity. Most studies to evaluate the effects of seismic survey focus on cetaceans (Gordon et al., 2003); however, commercial fish, rock lobsters and commercial scallops (Engås and Løkkeborg, 2002; Parry and Gason, 2006; Harrington et al., 2010) have also been the subject of research. Seismic airguns produce physiological stress in invertebrates and vertebrates, causing significant increases in mortality in copepods, (Fields et al., 2019), increases in hydrocortisone, glucose and lactate levels in golden carpet shell (La Bella et al., 1996), and mortality and damage to sensory systems in giant squid (Guerra et al., 2004). The effects of exposure to seismic airgun in most marine invertebrates remain unknown, with few studies and often limited due to experimental conditions (such as unknown sound exposure, artificial tanks or absence of controls; Carroll et al., 2017). Bioindicators are used to assess the impact of pollution on aquatic biodiversity and the methodology developed for one type of stress can be applied to other types (Chiaramonte et al., 2020; Lazzara et al., 2022; Mauro et al., 2021; Mauro et al., 2022).

During stressful conditions, the immune system plays a very important role and is a first line of defence to restore homeostasis. The immune system includes some important biomarkers such as total protein (TP), heat-shock protein levels, hydrolases and antioxidant enzymes (Weeks et al., 2018). Alterations in TP, for example, have been associated with various environmental stressors (Baden et al., 1990; Bjerregaard, 1991; Chen et al., 1994; Sánchez et al., 2001) and TP is commonly used to determine the physical condition of organisms. These biomarkers are critical indicators of the health of aquatic organisms, reflecting nutritional status, stress-response capacity, immune function, metabolism, homeostasis and the capacity for growth and reproduction (Ellis et al., 2011). Monitoring these levels can provide valuable information on the resilience of organisms to environmental stresses and their ability to adapt to changes in the environment (Bolton et al., 2009; Celi et al., 2013; Stewart and Li, 1969). Several protein components are involved in non-self-recognition, clotting and foreign agent elimination (Rosas et al., 2004), and heat shock proteins (HSPs) play a cytoprotective role: protecting cells from apoptosis and interacting directly with cell signalling pathways and apoptotic factors (Lanneau et al., 2008). The hydrolase class of enzymes, such as alkaline phosphatase and esterase, are known to play a key role in the removal of phosphate groups and ester bonds. Hydrolases are involved in the degradation of nutrients, the detoxification of harmful substances, the repair of cellular damage, the regulation of osmotic balance and the response to stress, thus ensuring the survival and health of organisms in variable and often hostile environments (Parisi et al., 2017; Vazzana et al., 2020a, 2020b). Antioxidant enzymes, such as peroxidase and superoxide dismutase, are crucial to restoring homeostasis by reducing oxidative stress and lowering levels of circulating reactive oxygen species (ROS). Although ROS are essential for many cellular functions, they can cause significant damage to DNA, proteins and lipids if produced in excess and lead to cellular dysfunction, inflammation, oxidative stress and cell death (Mydlarz and Harvell, 2007). Previous studies have analyzed the effects of different acoustic frequencies and intensities on vertebrate and invertebrate marine organisms, demonstrating significant changes in immune and behavioural responses (Buscaino et al., 2015; Buscaino et al., 2020; Mauro et al., 2020; Solé et al., 2023; Vazzana et al., 2020a, 2020b).

Many authors have studied biochemical parameters (including those described above) to evaluate the effects of acoustic stress on *Mytilus galloprovincialis*, *Palinurus elephas*, *Crassostrea virginica* and *Arbacia lixula*, highlighting significant changes in plasma glucose, protein levels, haemocyte count, enzymatic response (e.g., esterase, alkaline phosphatase and peroxidase), cytotoxicity activity, phenoloxidase activity, HSP27 and HSP70 expression (Celi et al., 2015; Vazzana et al., 2020a; Ledoux et al., 2023). *Arbacia lixula*, in particular, is characterized by a rigid, ovoid calcareous skeleton, containing the organs and surrounded by the peristomial membrane (Lawrence, 2013; Perricone et al., 2020). This membrane is a ligamentous structure that encircles the mouth of the sea urchin and wraps around Aristotle's lantern. Its morphology and physiology have been studied by Carnevali et al. (1990) and Bonasoro et al. (1995), who demonstrated a changeable, highly specialized structure. The collagenous membrane was shown to be capable of considerable deformability, consisting of a monolayer of columnar support cells mixed with sensory ciliated cells. The membrane also showed tensile properties, non-linear viscoelasticity, stiffness and elastic properties when subjected to vertical deformation (Wilkie et al., 1993). The aim of this study was evaluate, for the first time, the biochemical response of the peristomial membrane in sea urchin when exposed to seismic water gun shots by considering levels of protein concentration, esterase, alkaline phosphatase, superoxide dismutase (SOD), peroxidase and HSP70 expression.

2. Materials and methods

2.1. Animals and experimental setting

The experimental plan involved 28 adult individuals of *Arbacia lixula* (50 ± 8 g in weight and 4 ± 1 cm in diameter measured without thorns), which were manually collected in the natural environment by expert divers off the coast of Capo Granitola (Strait of Sicily, Italy). The animals were transported directly to a net cage at sea ($1.6 \times 0.8 \times 0.8$ m) for an acclimation period of four days before experimentation began. The cage (Fig. 1) was located at a depth of 8 m on a sandy bottom approximately 90 m off the coast (37° 34.225′ N–12° 39.360′ E). The experiment was carried out on 30th July 2018 and subdivided into 4 time slots: time t0 (1 h before water gun shots), time t1 (immediately following water gun shots), time t2 (3 h after shots) and t3 (24 h after shots). At each time slot, 7 sea urchin specimens were collected from the cage and transported rapidly to the laboratory for processing. Time t0 was considered as control treatment. The water temperature during the experimental day was monitored by 3-D particle displacement and a pressure sensitive recorder (see next paragraph for details), and ranged from 20.6 to 21.7 °C.

2.2. Water gun acoustic emission and analysis

The acoustic source used for the tests was a S15 Water gun (Sodera, France), a pneumatic seismic compressed air source. Table 1 shows the main characteristics of the acoustic source used. An autonomous 3-D particle displacement and pressure sensitive recorder (M20, Geospectrum ltd, Canada) was deployed 50 m from the source to characterize both the sea background and the acoustic signal produced by the seismic water gun at the precise location of the cage. During the test, the water gun position was maintained facing the recorder. Signals were emitted for a total of 20 min, with an interval of 8 s between one shot

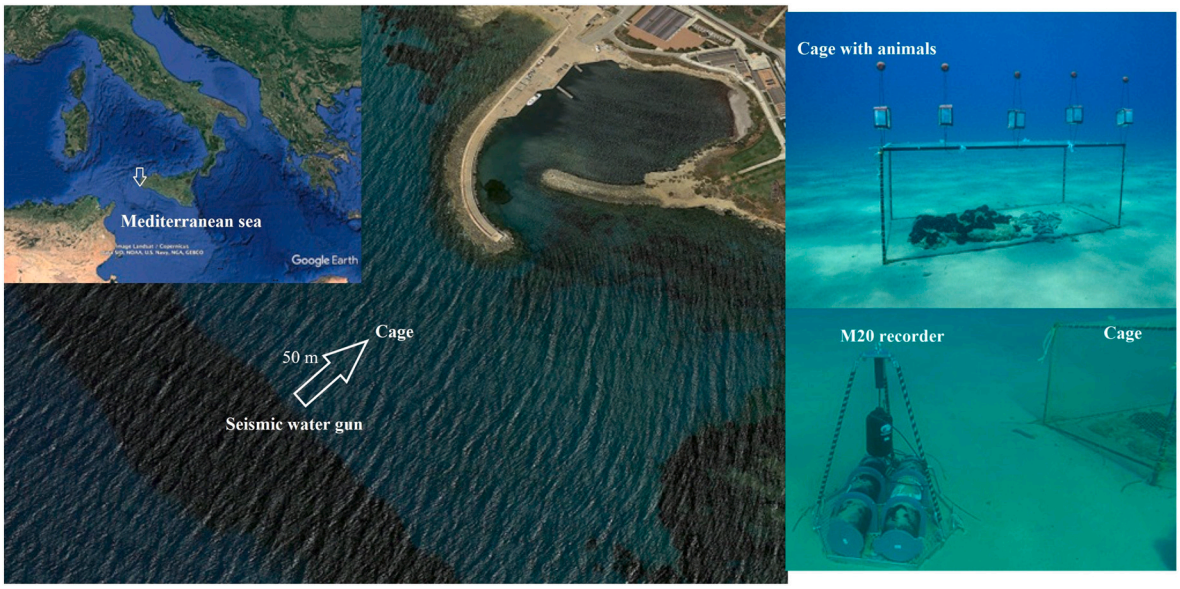

Fig. 1. The position of the cage within sea urchins on the bottom, and M20 recorder. In the insert, the location of Capo Granitola (Sicily Strait, Italy) laboratory was showed.

Table 1
Main characteristics of the acoustic source used (S15 water gun).

Material	Stainless steel
Length	516 mm
Width	152 mm
Weight	17.3 Kg
Air pressure	From 10 bars to 207 bars
Air requirement	0.16 l per shot
Firing cycle	0.25 s
Compressor requirement	87 Nm^3/h

and another. A total of 102 pulses were shot.

For each pulse detected, a 100 ms interval was considered: 20 ms before the impulse, and 80 ms after the impulse. For each pulse, the following parameters were estimated within the frequency band 1–2500 Hz:

- $L_{p,pk\text{-}pk}$ (dB re 1 μPa) - peak to peak sound pressure calculated in the time range where the cumulative energy is at 5 %–95 % of the maximum value;
- $L_{p,\ rms90\%}$ (dB re 1 μPa) - root mean square sound pressure calculated in the time range where the cumulative energy is at 5 %–95 %;
- $L_{u,\ rms}$ dB re 1 (dB re $nm/s)^2$ - root mean square sound particle velocity calculated in the time range where the cumulative energy is at 5 %–95 % of the maximum value for each component x, y, and z (direction bottom-surface) and velocity module. The frequency band is 1–2500 Hz.

- F_{peak} (Hz): Peak frequency is calculated as the maximum of the power spectrum curve.

2.3. Biological samples

At the end of each experimental point in time, the animals were removed manually, transported in closed containers filled with the same sea water as the natural site and supplemented with oxygen through an aeration stone and Tricaine methane sulfonate (MS-222) to anesthetize, as suggested by Applegate et al., 2016. The peristomial membrane around Aristotle's lantern was sampled in each individual using sterilized scissors and tweezers, and frozen at −20 °C. Each sample was thawed and homogenated using a glass piston homogenizer on ice with 900 μl of RIPA buffer 1× supplemented with a cocktail of protease inhibitors (1:200). Each sample was then sonicated for 1 min and centrifuged at 6800 rpm for 30 min at 4 °C. The supernatant was collected and stored at −20 °C in different aliquots (150 μl) for subsequent analysis.

2.4. Total protein

Total Protein (TP) was measured using the Bradford method (Bradford, 1976), with three replicates for each sample. Absorbance (Abs) was evaluated at 595 nm.

2.5. Enzymatic assays

Enzyme activities were measured, with three replicas for each sample. Superoxide dismutase (SOD) activity was determined using an assay kit for SOD determination (Sigma-Aldrich Product number 19160). The samples were incubated using a 96-well plate following manual instruction. Abs was measured at 450 nm using a microplate reader (GloMax; Promega Corporation, USA) and elaborated based on the formula:

$$SOD\ activity\ (\%) = \{[(Ablank1 - Ablank3) - (Asample - Ablank2)]/(Ablank1 - Ablank3)\}*100$$

where: *Ablank1*, *Ablank3* and *Ablank2* are the absorbance values obtained by incubating the solutions in the SOD kit. *Asample* was the absorbance value obtained by the sample. Using an inhibition curve (prepared by WSZ-1 assay with different incubation times), the concentration of SOD (U/ml) was obtained.

Peroxidase activity was measured using slight modifications to the Quade and Roth method (Quade and Roth, 1997). Each sample (50 µl) was incubated with 100 µl of TMB (3.3′, 5.5′ tetramethylbenzidine) (Sigma, Italy). The reaction was stopped after 30 min using 50 µl of 2Msulfuric acid (H_2SO_4) and Abs was measured at 450 nm. One unit of activity was defined as the amount of enzyme required to release 1 µmol of substrate produced in 1 min. Peroxidase activity was expressed as unit U/µg.

Esterase activity was evaluated incubating 50 µl of sample with 50 µl of buffer (0.4 mM *p*-nitrophenyl-myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5 % Triton X-100, pH 7.8, 30 °C) (Ross et al., 2000). Enzymatic activity was measured for each sample in three replicates. Abs was read at 405 nm for 1 h every 5 min.

Alkaline phosphatase activity was measured as suggested by Ross et al. (2000), incubating 50 µl of sample with 50 µl of buffer (4 mM *p*-nitrophenyl liquid phosphate in 100 mM ammonium bicarbonate containing 1 mM $MgCl_2$, pH 7.8, 30 °C). Enzymatic activity was measured for each sample in three replicates. Abs was read at 405 nm every 5 min for 1 h.

Esterase and alkaline phosphatase activities were expressed in U/µg and calculated as:

$$\{(\mathrm{Abs}/min) \times (1000/\mathrm{Eb}) \times (\mathrm{Vf}/\mathrm{Vi})\},$$

with $Eb = 16.4$ for esterase activity and $Eb = 18.4$ for alkaline phosphatase activity.

One unit of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol produced in 1 min.

2.6. SDS-PAGE and western blot

HSP70 protein expression was performed using western blot analyses (Towbin et al., 1979). The equivalent of 10 µg/ml for each sample were separated on 7.5 % SDS-PAGE gels at 60 V for the first 10 min and at 120 V for 1 h. Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in a transfer buffer (48 mM tris, 39 mM glycine, 20 % v/v methanol, pH 8.3) and a wet transfer apparatus (Bio-Rad, Mini-Protean II Cell) at 15 V for 1 h; correct transfer was confirmed by Ponceau red-staining. HSP70 was detected by overnight incubation at 4 °C with mouse HSP70 antibody (primary antibody dilution was 1:7500, Sigma-Aldrich). PVDF blots were washed twice with TBS-T 1× and incubated with secondary antibody alkaline phosphatase-conjugated goat anti-Mouse IgG (1:3000 dilution, Sigma-Aldrich). Development was finally carried out with BCiP-NBT. Densitometric analysis of the immunoblotted bands was performed using ImageJ software. Densitometry data were expressed as the mean values of different experiments and reported as a percentage of the Integrated Density Value (I.D.V.).

2.7. Statistical analysis

Each biochemical parameter (Total Protein, peroxidase activity, SOD activity, esterase activity, alkaline phosphatase activity and HSP70 expression) was tested for normal distribution using Shapiro-Wilk's test. As data were not normally distributed, the non-parametric Kruskal-Wallis test was applied to compare experimental times (t0, t1, t2, t3). Post-hoc multiple comparisons of mean ranks for all groups (Siegel and Castellan, 1988) were applied for each biochemical parameter which differed between time frames following the Kruskal-Wallis test.

In order to evaluate which time frame most affected the different biomolecular parameters, a generalized additive model (GAM) (R "mgcv" package version 1.8–28, Wood, 2012) was used. Each biomolecular parameter was modelled as a function of the time frame. A Gaussian distribution and identity link function were chosen and residuals from each model were assessed. Fairly constant variance, with only slight departures from the expected distribution, confirmed data fitting. GAM analyses were performed in R2022.02.0.

Table 2
Characterization of water gun shots (no. 120) at 50 m distance using M20 recordings.

Acoustic parameters	(Mean ± standard deviation)
$L_{pk,\ pk}$ (dB re 1 µPa)	178.2 ± 1.0
$L_{p,\ rms90\%}$dB re 1 $(nm/s)^2$	158.8 ± 2.3
$L_{u,\ rms\text{-}x}$ dB re 1 $(nm/s)^2$	231.4 ± 0.8
$L_{u,\ rms\text{-}y}$ dB re 1 $(nm/s)^2$	227.2 ± 0.9
$L_{u,\ rms\text{-}z}$ dB re 1 $(nm/s)^2$	232.6 ± 1.2
F_{peak} (Hz)	694.6 ± 82.0

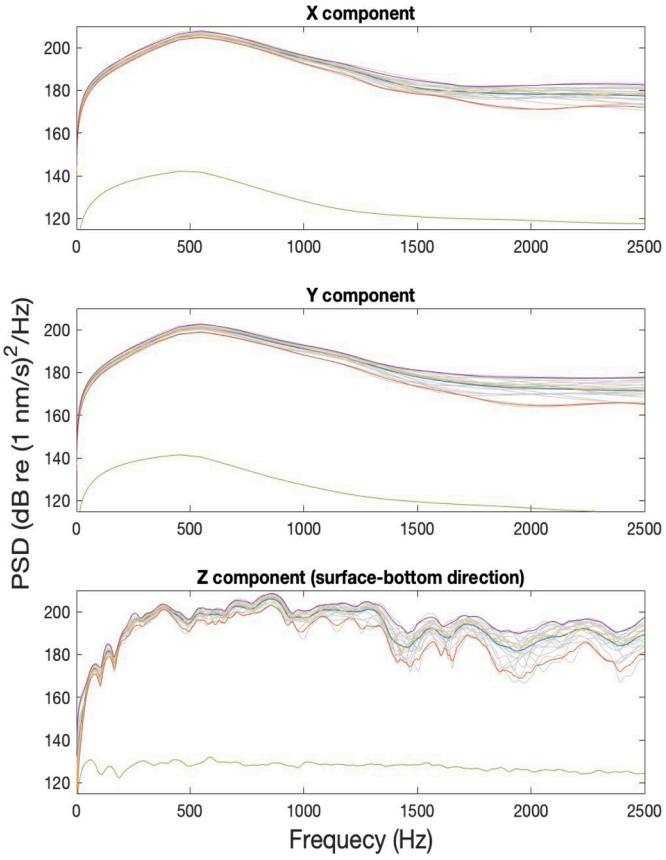

Fig. 2. Power Spectral Density of particle speed in the three direction X, Y, Z measured at 50 from the source close to the cage within the sea urchins. Total number of shots 120. Green line is the average background noise. Median, 95th and 5th percentile are expressed respectively in blue, violet and red lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Water gun acoustic characterization

The main acoustic measurements (mean ± standard deviation) of the 120 shots are shown in Table 2. The power spectral density of particle speed in three directions X, Y, Z measured at 50 from the source (close to the cage with the sea urchins) is shown in Fig. 2.

3.2. Biochemical parameters

A decrease in total protein (although not significant) was observed at the end of acoustic emission (t1); total protein subsequently returned to levels similar to the control (Fig. 3). A significant difference, however,

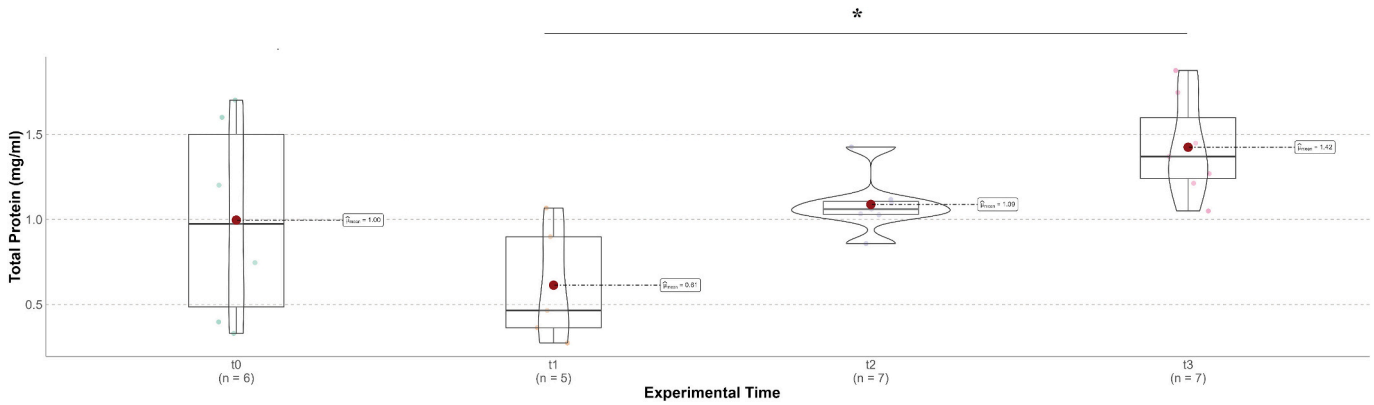

Fig. 3. Combination of violin plot, box plot (with 95 % lower and upper confidence intervals) and jittered data points of Total Protein of peristomial membrane of *A. lixula* at different experimental times: before water gun emission (t0), at the end of emission lasting 20 min (t1), 3 h (t2) and 24 h (t3) after the end of the water gun emission. Values were expressed in mg/ml. Red points indicate the mean value and asterisk indicates significant difference in multiple comparison test (see Table 3). Graphs are realized using R function "ggbetweenstats" (package "ggstats plot", Patil, I. (2021)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

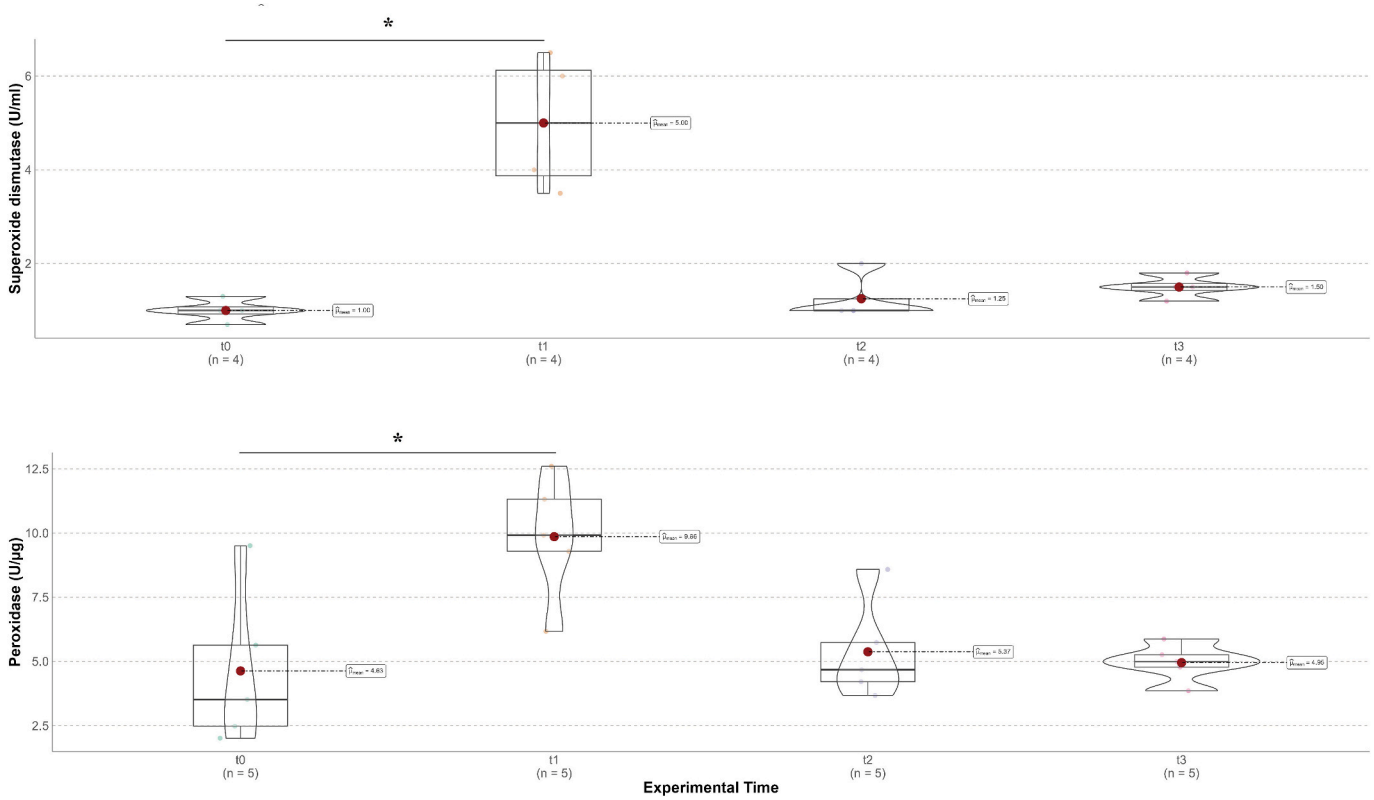

Fig. 4. Combination of violin plot, box plot (with 95 % lower and upper confidence intervals) and jittered data points of Superoxide dismutase (up) and Peroxidase activity (down) evaluated in peristomial membrane of *A. lixula* at different experimental times: before water gun emission (t0), at the end of emission lasted 20 min (t1), 3 h (t2) and 24 h (t3) after the end of water gun emission. Values were expressed in U/ml. Red points indicate the mean value and asterisk indicates significant difference of multiple comparison test (see Table 3). Graphs are realized using R function "ggbetweenstats" (package "ggstats plot", Patil, I. (2021)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was observed between t1 and t3 (KW-test: $p < 0.03$; GAM: $p = 0.052$, Dev. Explained: 40 %).

Superoxide dismutase (SOD) activity increased significantly (KW-test: $p < 0.02$; GAM: $p < 0.001$, Dev. Explained: 85 %) at the end of acoustic emission (t1); enzyme activity subsequently returned to control values (t0) both at time t2 and t3 (Fig. 4, Tables 3 and 4).

Significant increases in peroxidase activity (KW-test: $p < 0.03$; GAM: $p < 0.02$, Dev. explained 54 %) were found at the end of acoustic emission (t1) compared to the control (t0), while enzyme activity reached control values again at time t2 and t3 (Fig. 4, Tables 3 and 4).

Esterase activity significantly increased in t1 compared to the control (t0) (KW-test: $p < 0.002$; GAM: $p < 0.02$). After 3 h of acoustic emission (t2), esterase levels remained higher than the control, although the result was not significant. 24 h after the end of the water gun acoustic emission (t3), esterase activity was similar to control (t0), and lower than in t1 (KW-test: $p < 0.03$) (Fig. 5, Table 3).

Alkaline phosphatase activity showed an increase at time t1 (GAM: $p < 0.005$, dev. explained 46 %) compared to t0 (Fig. 5, Tables 3 and 4).

Table 3
Kruskal-Wallis and post-hoc multiple comparisons of mean ranks (Siegel and Castellan, 1988, p. 213–215) results comparing each biochemical parameter between different time phases (t0, t1, t2, t3). Red values indicate significant differences ($p < 0.05$).

	Multiple comparison, p values				Biochemical parameter	Kruskal-Wallis test
	to	t1	t2	t3		
t0		0.999	1.000	0.493	Total Protein	p=0.0208, n=25
t1	0.999		0.81	**0.012**		
t2	1.000	0.81		0.488		
t3	0.493	**0.012**	0.488			
t0		**0.014**	1.000	0.825	Superoxide dismutase	p=0.0113, n=16
t1	**0.014**		0.07	0.713		
t2	1	0.069		1.000		
t3	0.825	0.713	1.000			
t0		**0.028**	1.000	1.000	Peroxidase activity	p=0.0234, n=20
t1	**0.028**		0.129	0.149		
t2	1.000	0.129		1.000		
t3	1.000	0.149	1.000			
t0		**0.001**	0.097	1.000	Esterase activity	p=0.0008, n=20
t1	**0.001**		1.000	**0.020**		
t2	**0.001**	1.000		0.652		
t3	1.000	**0.02**	0.652			
t0		0.0834	0.585	1.000	Alkaline phosphatase	p=0.0581, n=20
t1	0.084		1.000	0.288		
t2	0.585	1.000		1.000		
t3	1.000	0.288	1.000			
t0					HSP70	p=0.1217, n=16
t1						
t2						
t3						

Table 4
Results of the Generalized Additive Models tested for each parameter (only significant results).

	Deviance explained	Variable	Estimates	Standard error	T value	P
Total protein	39.90 %	Time 3	0.42	0.21	2.05	0.052
SOD	84.90 %	Time 1	4,00	0.56	7.10	<0.001
Perossidasi activity	53.60 %	Time 1	5.23	1.40	3.73	0.001
Esterasi activity	76.40 %	Time 1	19.99	2.93	6.82	<0.001
		Time 2	10.55	2.93	3.59	0.002
Alkaline fosfatasi	46.10 %	Time 1	9.96	3.05	3.26	0.004
HSP70	39.00 %	Time 1	6.96	3.27	2.12	0.054

Enzyme activity reached control values 3 h after the end of the acoustic emission (t2) and remained similar until 24 h after emission (t3).

HSP70 expression did not show significant changes during experimental times, despite the fact an increase was detected at the end of water gun emission (t1) compared to the control (t0) (Fig. 6 and Table 4).

4. Discussion

In this study, Total Protein (TP), enzyme activity (esterase, alkaline phosphatase, peroxidase, and SOD), and HSP70 expression were evaluated in the peristomial membrane of *A. lixula* exposed to seismic water gun acoustic emission. To obtain realistic results and create a reference for the impacts of this activity on marine species, experimental tests were carried out at sea. Realistic noise pollution at lower frequencies is difficult to reproduce inside a tank. Furthermore, conducting the experiment on site allowed us to reduce the indoor confinement of individuals, thus partially reducing stress conditions (Bose et al., 2019).

Our results showed low TP levels at the end of acoustic water gun emissions (t1). 24 h after emission ended, TP significantly increased, returning to values similar to those of the control individuals. Total protein levels are important biomarkers in aquatic invertebrates for reasons related to physiology, stress response and adaptive capacity. TPs are important indicators of health and nutritional status as they reflect the balance between protein synthesis and protein degradation (Hook et al., 2014; El-SiKaily and Shabaka, 2024). The fall in total protein levels found in our study could indicate impaired protein synthesis or increased protein degradation, thus suggesting possible nutritional or metabolic problems caused by acoustic stress. Further confirming this point, as proteins can act as an energy reserve in conditions of stress, decreases in total protein levels may also be an indication that the organism is using proteins to satisfy energy needs. This occurs in a situation of food deficiency, probably due to a disturbance in the normal functioning of the peristomial membrane. Decreases observed following exposure to noise are also reported in other studies that evaluated the effects of acoustic stress on haemolymphatic and plasma samples of *Procambarus clarkii*, *Sparus aurata* and *A. lixula* (Celi et al., 2013; Celi et al., 2015; Vazzana et al., 2020b). However, TP is a highly variable

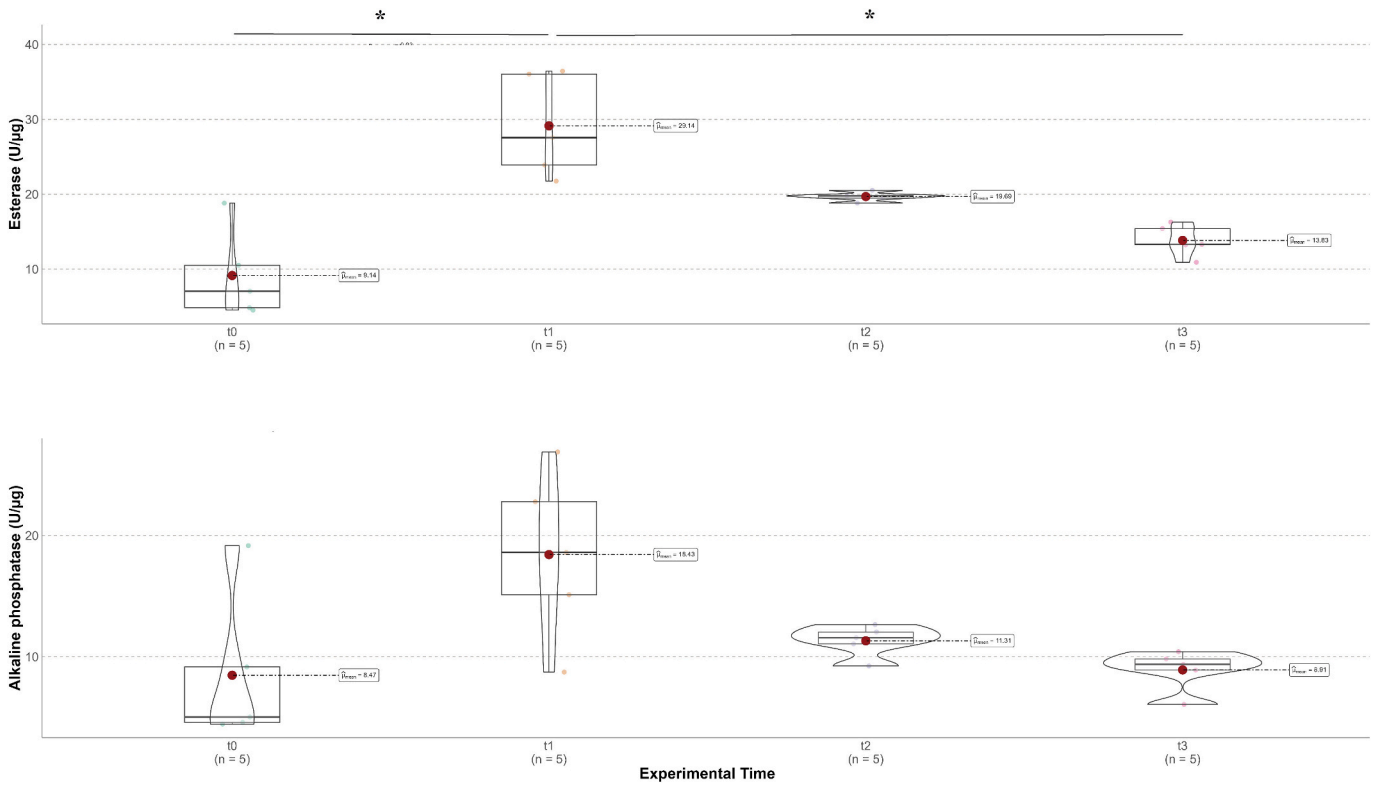

Fig. 5. Combination of violin plot, box plot (with 95 % lower and upper confidence intervals) and jittered data points of Esterase activity (up) and Alkaline phosphatase activity (down) in peristomial membrane of *A. lixula* at different experimental times: before acoustic emission (t0), at the end of emission lasted 20 min (t1), 3 h (t2) and 24 h (t3) after the end of water gun emission. Values were expressed in U/µg. Red points indicate the mean value and asterisk indicates significant difference of multiple comparison test (see Table 3). Graphs are realized using R function "ggbetweenstats" (package "ggstats plot", Patil, I. (2021)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

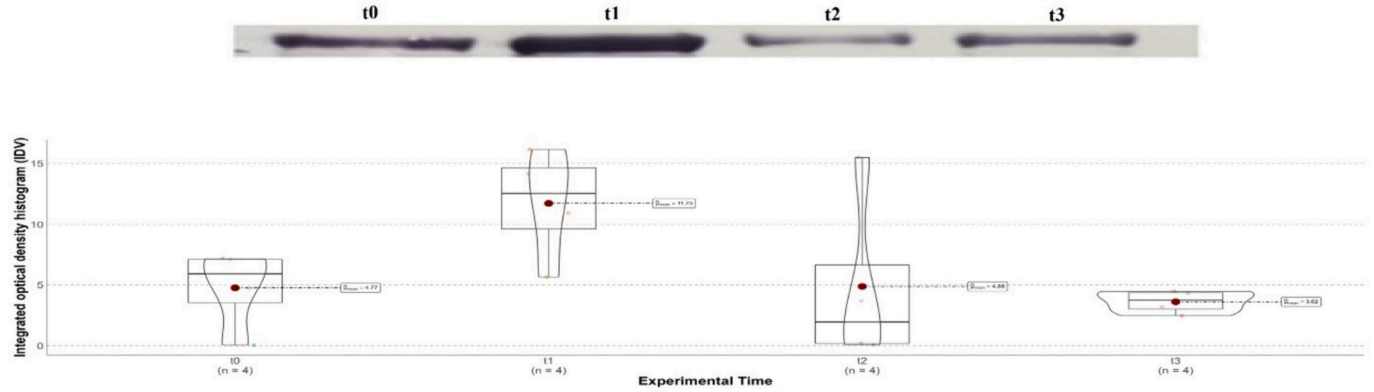

Fig. 6. Combination of violin plot, box plot (with 95 % lower and upper confidence intervals) and jittered data points of Integrated optical density histogram (IDV) of the HSP70 protein bands evaluated in peristomial membrane of *A. lixula* at different experimental times: before water gun emission (t0), at the end of emission lasted 20 min (t1), 3 h (t2) and 24 h (t3) after the end of water gun emission. Graph is realized using R function "ggbetweenstats" (package "ggstats plot", Patil, I. (2021)).

parameter in invertebrates. Levels can depend on species and genus, on environmental conditions and even on the age of individuals (Celi et al., 2015; Coeurdacier et al., 2011; Vazzana et al., 2017; Weinstock, 2011). Thus the limited number of individuals involved in the experiment could explain the absence of strong statistical differences.

HSP70 showed an increasing trend ($p = 0.054$, see Table 4) at the end of acoustic emission. The increase in heat shock proteins under stress conditions represents a fundamental mechanism for cell survival, as these proteins are activated to protect cells from irreversible damage. Their increased expression under conditions of acoustic stress could indicate the need to implement cellular defence mechanisms (Hu et al., 2022; Kumar et al., 2022). In stressful situations, such as acoustic stress, cellular proteins can denature or fold incorrectly, compromising cellular function. This situation would stimulate increases in levels of HSPs, which act as a kind of rescuer or protein chaperone, deployed to repair or eliminate damaged proteins and maintain cellular homeostasis (Hu et al., 2022). HSPs assist the correct folding of proteins, prevent protein aggregation, facilitate the repair of damaged proteins and contribute to maintaining proteins in a functional and stable state (Almalki et al., 2021). Furthermore, HSPs not only protect existing proteins but can also activate other cellular defence mechanisms or processes to repair damaged DNA (Kumar et al., 2022). All these aspects are fundamental to

ensure that cells maintain their integrity and functionality, evidently compromised under conditions of acoustic stress. Although HSP levels in the peristomial membrane had not been evaluated prior to our study, increases in HSP27 proteins in haemocyte lysates and HSP70 in the haemolymph of other crustacean species exposed to acoustic stress have been reported previously (Celi et al., 2013). Furthermore, Vazzana et al. (2017) reported that noise exposure caused increases in HSP70 levels in fish species.

Our results showed significant increases in peroxidase activity and SOD at the end of 20 min of acoustic emission. SOD and peroxidase are key antioxidant enzymes that protect cells by neutralizing ROS (Hong et al., 2024). The results obtained suggest that acoustic stress may generate oxidative stress within the cells of the peristomial membrane of sea urchins. Enzymatic increases indicate that cells are trying to counteract excess production of ROS, which would otherwise damage cellular biomolecules such as proteins, lipids and DNA (Juan et al., 2021). Increases in enzyme activity can, therefore, be interpreted as an adaptive defence response in cells to acoustic stress. The significant increases observed suggest, therefore, that acoustic stress is causing cell damage and that the antioxidant defence system is activated as a consequence. Antioxidant enzymes have been evaluated in the tissue of other sea urchin species, such as *Lytechinus variegatus*, *Strongylocentrotus franciscanus* and *Strongylocentrotus purpuratus* (Du et al., 2013). However, no one had evaluated to date the effects of acoustic stress on antioxidant activity in the peristomial membrane. In our case, increases in SOD levels suggest an increase in oxidative stress with removal of O_2, and production of H_2O_2, subsequently removed by peroxidase enzyme activity. (Bogdan et al., 2000). Furthermore, the peroxidase activity found in this study was comparable to a previous study of ours in which the activity of this enzyme was evaluated in the coelomic fluid of *A. lixula* exposed to acoustic stress (Vazzana et al., 2020b).

In addition to antioxidant enzymes, enzyme levels in the class of hydrolases were also evaluated. Esterase activity increased significantly immediately following the end of seismic water gun emission. Alkaline phosphatase levels increased in t1 ($p = 0.004$, see Table 4). Esterase and phosphatase enzymes, as in the cases above, may be involved in cellular defence mechanisms and invertebrate adaptation to environmental stress, including acoustic stress (Rodrigues et al., 2014; Vazzana et al., 2020a, 2020b). Linked to earlier observations on total proteins, increased activity in these enzymes could reflect the metabolic adaptation of cells to face new conditions imposed by acoustic stress, for example, by modifying the rate of hydrolysis of cellular substrates (Rothman et al., 2023). Lastly, these enzymes could be involved in the detoxification of harmful chemical substances or in the metabolism of toxic compounds accumulated following acoustic stress (Morisseau, 2022).

Numerous studies show variations (both positive and negative) in levels of hydrolase enzymes in invertebrates subjected to stress conditions; however, no investigations had been carried out on the peristome of sea urchins. Parisi et al. (2017) demonstrated that under conditions of high temperature and anoxia, enzyme activity in the mussel gland decreases. Goswami et al. (2014) demonstrated a decrease in esterase activity in shellfish tissue exposed to Cu and Cd, but an increase when exposure was combined. High temperatures, pH, and viscera regeneration can also cause significant changes in alkaline phosphatase levels (Hu et al., 2015; Zang et al., 2012). Comparable with our results, Vazzana et al. (2020b) observed significant increases in these enzyme levels in coelomic fluid of *A. lixula* exposed to acoustic stress. Biomarker expression can vary according to species (Brown et al., 2004) and tissues being analyzed (Forget and Bocquene, 1999; Franco-Martinez et al., 2016). Increases in the hydrolase enzymes found in our study could indicate not only compromised metabolic rates but also an inflammatory state in the peristomial membrane. This may derive from a "sounding board" effect in the skeleton of the sea urchin, triggered by noise through water and the substrate (Radford et al., 2008).

Despite the important results obtained in this study, future research should increase the number of individuals used per time point, expand the biomarkers analyzed as a consequence of stress, and also consider molecular aspects and the possible timing of restoration of homeostasis. Although echinoderms produce sounds during grazing (Radford et al., 2008) and a couple of studies demonstrated that they are affected by noise (Vazzana et al., 2020a, 2020b; Solan et al., 2016), there is currently a knowledge gap regarding their ability to perceive sounds both in terms of pressure variations or particle movement. Marine invertebrates have cilia-based mechanosensory (hair cells) or statocyst receptor systems allowing them to detect underwater sound (Solé et al., 2023). The sensory hair cells and statocyst are solicited by mechanical processes that are activated by particle motion; whereas membranes and gas-filled cavities respond mostly to pressure variations. Due to the fact that marine invertebrates generally do not have gas-filled bladders (Solé et al., 2023; Carroll et al., 2017), they undoubtedly sense mainly as a result of particle motion. Sea urchins have statocyst and mechanosensory organs in tube feet, mainly involved, it would seem, in sense of gravity and rheotaxis (Garm, 2017). Moreover, areas of greatest touch-sensitivity are located in the sensory hillocks, located near the jaws of the pedicellariae (Campbell and Laverack, 1968). These structures, responding to hydrodynamics and to direct touch with the substrate, could be sensitive to particle motion caused by sound; however, future studies are needed to confirm this mere hypothesis.

In conclusion, the biochemical responses analyzed in this study in the peristomial membrane of the sea urchin show a high level of response to acoustic disturbances, making this tissue a possible candidate for evaluating the effects of anthropogenic emissions. Our data showed a return to the control condition (t0) 24 h after water gun exposure. However, longer and more intense exposure could have a severe impact by impairing other biological activities, such as food acquisition, regulation due to environmental perturbations, migration and reproduction. Increases in almost all the parameters which were analyzed suggest substantial effects on the functionality of the membrane and on the nutritional capacity of the animals, in particular. It is evident that any compromise in the nutrition of the individual could have consequences on populations and, therefore, on the marine ecosystems in which they live (Devlin and Brodie, 2023; Malone and Newton, 2020). We could witness alterations in benthic communities in which sea urchins play a crucial role in controlling algal populations, in fact, if their density were to decrease due to malnutrition, algae could proliferate at an uncontrollable rate, altering the balance of the ecosystem (Steneck, 2013). Consequent increases in algae could harm other benthic life forms, thus reducing biodiversity. Furthermore, many marine predators, such as fish and crustaceans, feed on sea urchins (Pinna et al., 2024). A reduction in sea urchin populations could reduce the availability of food for these predators, negatively affecting their health and populations, and, ultimately, ecosystems (Steneck, 2020). Sea urchins are a key component of marine food webs (Lawrence, 2013). Their malnutrition and the resulting effects on populations could disturb food chains, leading to imbalances and potential structural changes in the ecosystem.

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CRediT authorship contribution statement

M. Mauro: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **M. Vazzana:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **M. Ceraulo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **C. de Vita:** Writing – review & editing, Formal analysis. **V. di Fiore:** Investigation. **V.M. Giacalone:** Writing – review & editing, Investigation. **R. Grammauta:** Writing – review & editing, Methodology, Investigation, Formal analysis. **V. Lazzara:** Writing – review & editing, Methodology. **E. Papale:** Writing – review & editing, Investigation, Formal analysis. **A. Vizzini:** Writing – review & editing, Methodology. **G. Buscaino:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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