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Active mussel biomonitoring for the health status assessment of the Western Mediterranean Sea

Denis Benito [a,c], Marine Briand [b], Olivier Herlory [b], Urtzi Izagirre [a,c,*], Marc Bouchoucha [b], Tifanie Briaudeau [a,c]

[a] CBET+ Research Group, Department of Zoology and Animal Cell Biology, University of the Basque Country (UPV/EHU), Leioa, Basque Country, Spain
[b] Ifremer, Lab Environ Ressources Provence Azur Corse, CS 20330, F-83507 La Seyne Sur Mer, France
[c] Research Centre for Experimental Marine Biology and Biotechnology (Plentzia Marine Station; PiE-UPV/EHU), University of the Basque Country, Plentzia, Basque Country, Spain

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ABSTRACT

The Western Mediterranean coast is under the influence of anthropogenic pressures, including land use, increasing amounts of dangerous waste and habitat destruction. In 2021, the French RINBIO network (http://www.ifremer.fr/envlit/) originally dedicated to assess chemical contamination in the region, focused on biological effects produced by contaminants and the interaction with natural variability in mussels using an active caging strategy. Cell and tissue level biomarkers were applied for 17 sampling sites divided in three sub-regions categorized by different environmental conditions. Results provide critical information for ecosystem health assessment using mussels as sentinel species in the Western Mediterranean Sea. The influence of natural and confounding factors (trophic condition, reproductive cycle, caging strategy), on biological responses to mild chemical contamination, was discussed and discriminated for health status assessment. Results provide valuable data available as reference values for the assessment of biomarkers and histopathological alterations for large-scale active biomonitoring campaigns in the Western Mediterranean Sea.

1. Introduction

Marine environments are subject to various disturbances including inputs of numerous chemical compounds, which have the potential to affect cellular and physiological processes in exposed organisms, as well as fundamental processes in ecosystems (Fleeger et al., 2003; Hylland et al., 2006b). In the past, European countries with a coastline implemented monitoring programs targeting chemical concentrations in biotic and abiotic matrices (Roose and Brinkman, 2005; Quevauviller et al., 2011). Their early objectives were to prevent contaminant consumption for human health and to quantify the presence and spatial extent of elevated concentrations of selected contaminants for environmental regulatory purposes (Farrington et al., 1983; Goldberg et al., 1983; Martin, 1985). The assessment of biological effects to understand contaminant impacts in marine ecosystems only started in the 1980s (Bayne, 1988). In the late 1990s, OSPAR signatory countries implemented contaminant-monitoring programs which included contaminant related biological effects (OSPAR, 1998a, 1998b). The concept of the biological approach was discussed in many scientific publications (Vethaak and Ap Rheinallt, 1992; Depledge et al., 1993; Hylland, 2006; Hylland et al., 2006a; Laane et al., 2012) and in international workshops (particularly ICES WGBEC). Over the past two decades, techniques focusing on selected biological effects including biomarkers were developed, validated, and guidelines were subsequently established for international organizations with a monitoring role, i.e. OSPAR, HELCOM and MEDPOL (Roose et al., 2011). These procedures and strategies were recently carried over into the implementation of the Marine Strategy Framework Directive (MSFD, e.g. Thain et al., 2008; Law et al., 2010; Lyons et al., 2010, 2017; Burgeot et al., 2017; Vethaak et al., 2017), and an international workshop on marine integrated contaminant monitoring (ICON) was initiated to test the framework above mentioned in practice on a Europe-wide scale, in the North Sea initially and extended to the Baltic, France (Seine Bay) and Spanish Mediterranean waters (Hylland et al., 2017).

Mussels (*Mytilus* spp.) are widely used as sentinel species in biomonitoring programs to survey environmental concentrations of pollutants and to assess the general health status of coastal and estuarine ecosystems (Widdows et al., 2002; Benedicto et al., 2011; Fernández

* Corresponding author at: Areatza Pasealekua, 48620 Plentzia-Bizkaia, Basque Country, Spain.
E-mail address: urtzi.izagirre@ehu.eus (U. Izagirre).

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et al., 2012; Marigómez et al., 2013a; Sturludottir et al., 2013; Farrington et al., 2016; Beyer et al., 2017; Santos-Echeandía et al., 2021; Benito et al., 2023; Briand et al., 2023). Alterations of their general health status are considered indicative of biological effects of surrounding stressors, including contamination, and are assessed based on biological-effect approaches at different levels of biological complexity (Izagirre and Marigómez, 2009; Garmendia et al., 2011; Brenner et al., 2014). Biological responses inform on changes in the general health status of an ecosystem, often interpreted as a reflection of contaminants effects but not solely. Indeed, biomarkers can be dependent on environmental conditions such as tidal regime, temperature and seasonality (Leiniö and Lehtonen, 2005; Benito et al., 2019) and biological traits such as individuals' reproductive status and food availability (Vethaak et al., 2011; Benito et al., 2019; Blanco-Rayón et al., 2019).

The Mediterranean coast historically receives a tremendous anthropogenic pressure, which includes extensive land use, decreasing freshwater resources, increasing amounts of sewage, litter and dangerous waste, habitat destruction and contamination of fishery/marine resources (UNEP, 1985, 1992, 1997). The French RINBIO network (http://www.ifremer.fr/envlit/) is a monitoring program dedicated to Mediterranean coastal waters that uses artificial caging to assess contaminants levels and bioavailability (Cossa, 1989; Riget et al., 1997; Andral et al., 2004). The implementation of cages throughout the coast is a key element to control the spatial monitoring strategy. Another strong advantage of this transplantation method is the control over the source, age and stage of sexual maturity of the sampled mussels, which are essential factors to be taken into account in ecosystem health assessment. However, its application on a large geographic scale introduces factors such as variations in physiochemical characteristics and trophic conditions in the immersion zones (Andral et al., 2004). Until 2018, these campaigns were dedicated to chemical analysis to assess contaminant bioavailability in mussels' tissues. In comparison, the last 2021 campaign (SUCHI Med, Herlory et al., 2021) follows the biological approach where, in addition to chemical analysis, biological effects of contamination are assessed based on the application of a battery of biomarkers. Individuals' health status was assessed based on a set of cell and tissue level biomarkers to reach different levels of biological complexity (Marigómez et al., 2013a; Benito et al., 2023).

The aim of the present study was to perform the health status assessment of the Western Mediterranean Sea using mussels (*Mytilus galloprovincialis*) as sentinel species. All within the context of a large-scale active biomonitoring program including multiple sampling sites along the French Mediterranean coast and performing an evaluation of the extent of the influence of environmental contamination and certain natural confounding factors on the measured biological responses.

2. Materials and methods

2.1. Sampling strategy

Mussels, *M. galloprovincialis*, were transported by boat to 66 stations distributed along 800 km of French Mediterranean shoreline during the SUCHI Med campaign (DOI 10.18142/291) (Fig. 1). Onboard, individuals were kept in a flow through system to maintain constant water conditions during transportation. Before starting the caging experiment, a control group was composed directly after collecting mussels from the

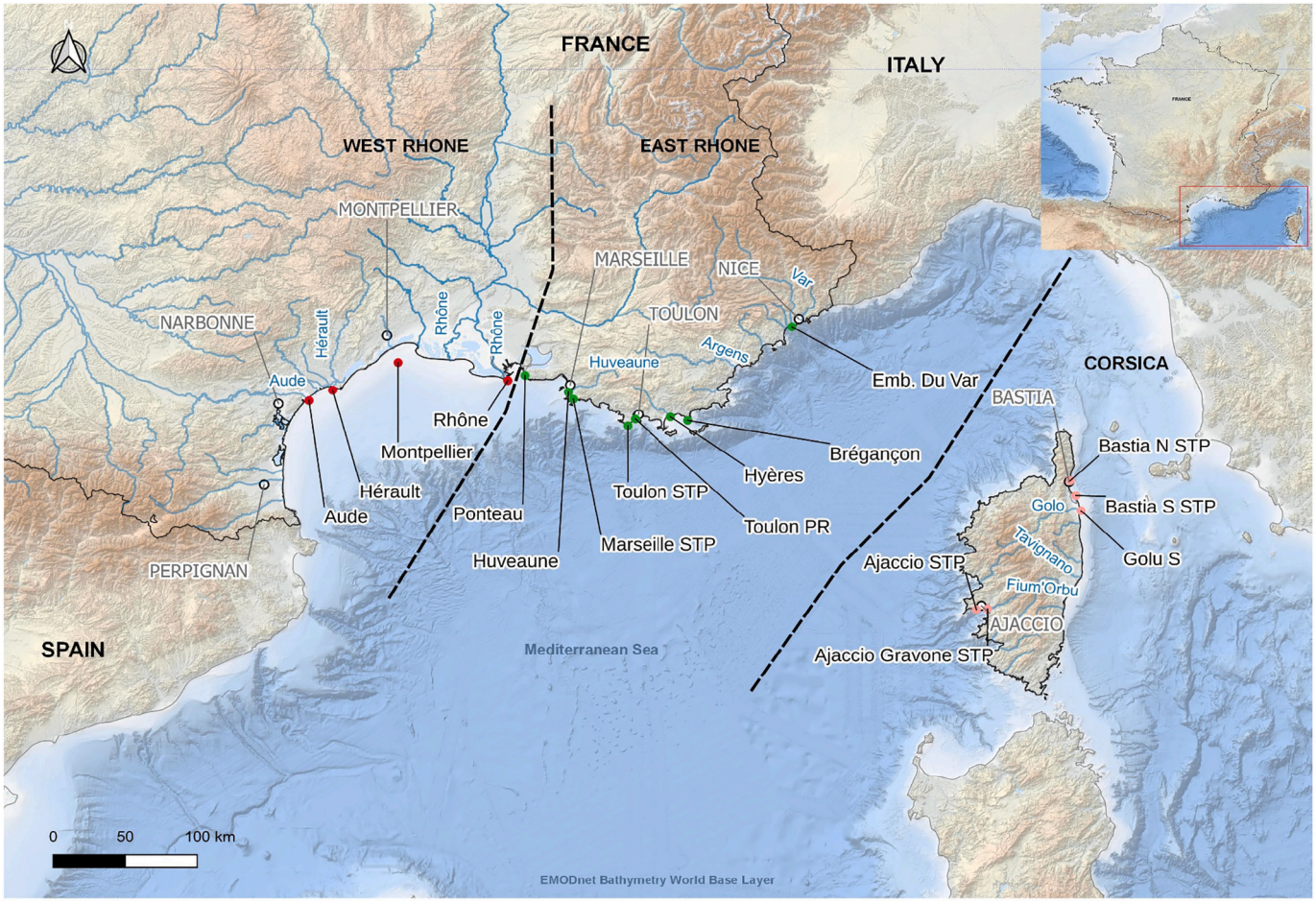

Fig. 1. Map of the French Mediterranean Sea in which the caging sites are marked in red for the West Rhône region, green for the East Rhône region and pink for Corsica. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

farm to assess the initial biological status of individuals ($n = 20$). Of the 66 stations, 17 were selected for biomarker analysis. These stations were specifically chosen for their localization near main sources of contamination, i.e. important rivers, big cities and water treatment plants. For interregional comparisons, three regions were defined: West Rhône, which included the sites from Aude to Rhône; East Rhône, which included the sites from Ponteau to Emb. Du Var and Corsica, which included the sampling sites around the island. At each station, 3 kg of 18–24 months old mussels (approximately 130–160 mussels) were placed in man-made RINBIO biointegrator network conchylicultural pouches and were immersed for 3 months in spring 2021 starting from mid-March to early April until mid-June to early July, depending on the sites (see Briand et al., 2023 for details). The RINBIO protocol is described in more details in Andral et al. (2004).

2.2. Trophic indicators and mortality

Several parameters measured on site or in mussels were used as indicators of trophic conditions: total concentration of chlorophyll *a,* (Chl-*a* concentration, $\mu g.L^{-1}$), isotopic ratios $\delta^{13}C$ and $\delta^{15}N$ (‰) and C/N ratio, fat content (FC, %) and condition index (CI).

The total Chl-*a* concentration was assessed by spectral analysis observed daily by colour sensors from different satellites (MODIS and OLCI) and estimated by the OC5 algorithm as developed by Ifremer (see Gohin et al., 2020 for method). The percentile 90 (P_{90}) values for the immersion period were calculated.

Isotopic ratios $\delta^{13}C$ and $\delta^{15}N$ (‰) and C/N ratio were measured in mussels tissue to estimate water trophic signature. For each station, 0.5 mg of ground and lyophilized mussel tissue ($n = 15–25$) was prepared in capsule for EA-IRMS analysis. Prepared samples were analysed by stable isotope ratio mass-spectrometry in continuous flow (Isoprime 100) paired with an elementar analyser (Elementar Vario Pyrocube). V-PDB (Vienna - PeeDee Belemnite) and atmospheric N_2 were used as international standards for carbon and nitrogen, respectively. Analysis were performed by the "Laboratoire d'Océanographie de Villefranche" (LOV) from Sorbonne University.

Fat content (FC, %) was analysed in mussels tissue (n = 15–25) by the laboratory LABERCA-ONIRIS (ISO/IEC 17025:2017-certified Quality Assurance), which was in charge of organic contaminant analyses of the campaign SUCHI Med (Briand et al., 2023). Lipids were extracted from freeze-dried tissues by pressurized liquid extraction (PLE SpeedExtractor - Buchi, France, internal method LABERCA/DGAl/DPCB-sah.1.01) with a mixture of toluene/acetone (70:30). The total lipid amount was determined gravimetrically.

For each sampling point, mortality was recorded for the entire pouch and 15–25 mussels per pouch were selected for biometric measurements (length, width, and height of the shell). In the laboratory, shells were dried in the oven at 60 °C for 48 h and weighed to determine the dry shell weight (SW). Mussel tissue was weighed before and after freeze-drying to obtain the dry tissue weight (W). Finally, individual condition index (CI) was calculated from the ratio W/SW and used as an indicator of mussels' physiological status and growth.

2.3. Lysosomal biomarkers

For each station, digestive glands ($n = 10$) were dissected out on site, directly frozen in liquid nitrogen and stored at −80 °C for histochemical analysis. Once in the laboratory, frozen samples were sectioned using a CM3050s Leica cryotome and stored at −40 °C until used for lysosomal biomarkers assessment. Lysosomal membrane stability (LMS) and lysosomal structural changes (LSC) were selected as general stress biomarkers (Marigómez et al., 2006; Izagirre et al., 2008). LMS was assessed in 10 μm frozen sections, as a measurement of the lysosomal membrane labilisation period (LP) (in min), after demonstration of hexosaminidase (Hex) activity in digestive cell lysosomes (UNEP/RAMOGE, 1999), as detailed in Benito et al., 2019. LSC were determined in 8 μm frozen sections, based on image analysis after histochemical demonstration of β-glucuronidase as described in Moore (1976). The stereological procedure applied for image analysis is detailed in Benito et al., 2019. Lysosomal structure measurements included lysosomal volume density (Vv_{LYS}), lysosomal surface-to-volume ratio (S/V_{LYS}) and lysosomal numerical density (Nv_{LYS}).

Intracellular neutral lipid accumulation is known to indicate biological effects of general stress factors, in particular the exposure to organic contaminants (Marigómez and Baybay-Villacorta, 2003). The volume density of neutral lipid (Vv_{NL}) was assessed in 8 μm frozen sections of mussel digestive glands after Oil Red O staining (Culling, 1974).

2.4. Histological processing

Cross sections containing mantle, gills and digestive gland ($n = 12$ per station) were collected for tissue-level biomarkers assessment and histopathological examination. Samples were fixed in 4 % neutral buffered formaldehyde for 24 h at 4 °C and transferred to 70° alcohol until further processed in the laboratory. Afterwards, samples were dehydrated in a graded series of ethanol (70°/96°/pure ethanol), cleared and embedded in paraffin (Leica ASP300S). Histological sections (5 μm) were obtained using a rotary microtome (Leica RM2125RTS) and stained with hematoxylin-eosin for histological examination (Nikon Eclipse E200).

2.5. Sex and gonad developmental stages determination

Individuals' sex and gamete developmental stages were determined according to Ortiz-Zarragoitia et al. (2011).

2.6. Epithelial thinning of the digestive alveoli (Atrophy index)

As an indicator of general stress, epithelial thinning of the digestive alveoli can be assessed based on the calculation of the atrophy index as described by Kim et al. (2006). The index uses 4 semi-quantitative stages where 0 describes an almost occluded lumen of the digestive diverticula and 4 describes an extreme case where most tubules are affected with particularly thin digestive epithelium.

2.7. Digestive gland integrity (Connective Tissue Index)

Digestive gland histological integrity is assessed as an indicator of exposure to general stress, including xenobiotics (Marigómez et al., 2006; Garmendia et al., 2011). It can be estimated based on the determination of the interstitial connective tissue to diverticula ratio (connective tissue index), as a representation of the proportion of digestive alveoli to interstitial connective tissue. Semi-quantification of the connective tissue index followed four scores as described by Fraga et al. (2022).

2.8. Mantle energy storage (ADG cell index)

The estimation of mantle energy reserve is used as an indicator of metabolic strategy in molluscs and can be estimated based on the presence of adipogranular (ADG) cells (Bignell et al., 2008; Benito et al., 2023). ADG cell density was assessed based on the method described by Bignell et al. (2008).

2.9. Histopathology

Histopathological examination included the determination of the presence of lesions and parasites as described by Bignell et al., 2008. For each of them, prevalence was calculated as the percentage of individuals showing the identified lesion or parasite ([number of cases/total cases analysed] × 100). Identified parasites included Turbellarians and

intracellular ciliates (IC). Histopathological lesions included haemocytic infiltration, brown cell infiltration in gonad and digestive gland, and follicular atresia.

2.10. Statistical analysis

Prior to statistical analysis, the normality and homogeneity of data on biomarkers were tested with the Shapiro-Wilk and Levene tests, respectively. Interregional and intraregional comparisons were tested using the non-parametric Kruskal-Wallis test (non-normally distributed data or semi-quantitative endpoints) or the parametric One-way Anova test (normally distributed data). Statistical differences with the control group were tested with the non-parametric Mann-Whitney *U* test. All statistical analysis on biomarker data were carried out using IBM SPSS Statistics Base 22.0, with a level of significance $p = 0.05$. Intraregional significant differences regarding trophic indicators and mortality were determined by *Z*-score test (p = 0.05).

3. Results

To aid with reading and interpretation of the Results and Discussion sections, a table summarizing the biological endpoints measured in the present work is available as supplementary material (S1).

3.1. Trophic indicators and mortality

In each region, the sites with the highest Chl-*a* concentrations were Rhône (2.13 μg/L) for West Rhône region, Toulon PR (2.03 μg/L) and Emb. du Var (1.94 μg/L) for East Rhône region and Ajaccio Gravone STP (1.69 μg/L) for Corsica. Mean isotopic signatures in mussels ($\delta^{13}C$ and $\delta^{15}N$) varied significantly between regions with the highest $\delta^{13}C$ (−20.90 ± 0.56 ‰) and lowest $\delta_{15}N$ (3.77 ± 0.18 ‰) detected in Corsica and the lowest $\delta^{13}C$ (−21.83 ± 0.44 ‰) and highest $\delta_{15}N$ (4.74 ± 0.38 ‰) detected in the West Rhône region. The $\delta^{13}C$ value for Emb. du Var was significantly higher than the other sites from the East region (−19.34 ‰). C/N ratio measured in mussel tissue differed between the three regions, with the lowest mean value recorded in Corsica (3.64 ± 0.60) and the highest in West Rhône (5.36 ± 0.40). When comparing the mortality rates intraregionally, the lowest percentage was detected in Toulon PR (4.4 %, East Rhône) and the highest in Bastia N STP (23 %, Corsica). CI and FC were significantly lower in Corsica (0.06 ± 0.01 and 0.71 ± 0.10 %, respectively), while highest mean values were recorded in West Rhône (0.12 ± 0.02 and 1.20 ± 0.15 %, respectively). In each region, CI from Huveaune for East Rhône (0.14) and from Bastia S STP for Corsica (0.07) were significantly higher than the other sites. FC of mussels from Toulon PR (1.42 %) was significantly higher than the other sites from the East Rhône region.

3.2. Lysosomal biomarkers

3.2.1. Lysosomal Membrane Stability (LMS)

When comparing LP values among regions (Fig. 2A), significantly higher levels were recorded in mussels from Corsica when compared to mussels from West Rhône and East Rhône. Statistical comparisons within groups in the West Rhône region showed that individuals sampled in Rhône and Aude displayed significantly higher values than mussels from Hérault. In Corsica, mussels from Ajaccio Gravone STP showed significantly higher values than the ones from Bastia S STP. Mussels from Hérault, Montpellier, Ponteau, Huveaune, Toulon STP and Bregançon displayed significantly lower LP values when compared to control mussels. Mussels from Ajaccio STP and Ajaccio Gravone STP displayed significantly higher LP values than the control mussels.

3.2.2. Lysosomal Structural Changes (LSC)

Overall, the lowest Vv_{LYS} regional values were recorded in mussels from Corsica whilst the higher levels were observed in mussels from the West Rhône and East Rhône regions (Fig. 2B). When comparing Vv_{LYS} values in different stations in the West Rhône region, mussels from Montpellier and Rhône showed significantly lower values. When comparing mussels from different stations in the East Rhône region,

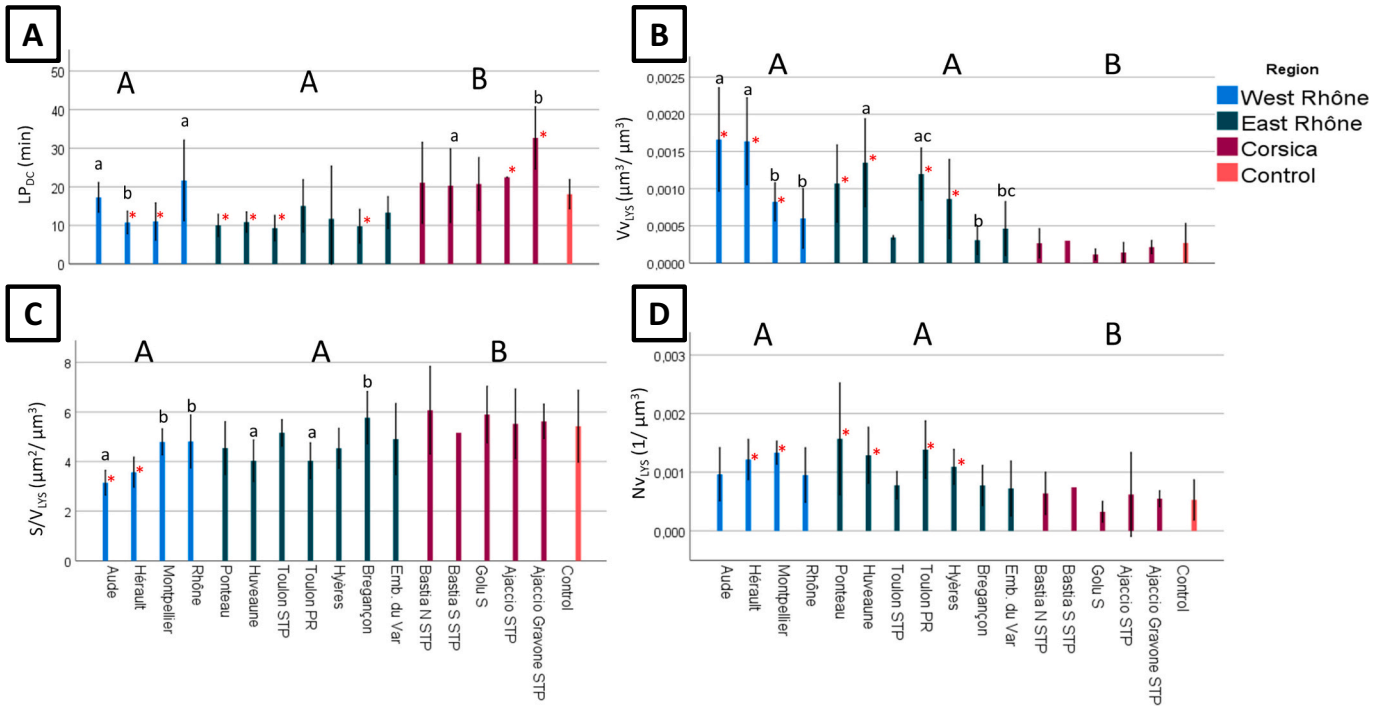

Fig. 2. A: Mean labilisation periods (LP) measured in Lysosomal Membrane Stability (LMS) test (min, $n = 10$). B: Mean lysosomal volume density (Vv_{LYS}, n = 10). C: Mean lysosomal surface/volume ratio (S/V_{LYS}, n = 10). D: Mean lysosomal numeric density (Nv_{LYS}, n = 10). Large letters mean statistical differences between regions, small letters indicate significant differences between sampling sites in the same region and asterisks indicate differences when compared to the control group ($p < 0.05$).

mussels from Huveaune presented significantly higher Vv_{LYS} values when compared to mussels from Emb. Du Var and Bregançon, while mussels from Toulon PR presented significantly higher Vv_{LYS} values when compared to mussels from Bregançon. Mussels from Aude, Hérault, Montpellier, Ponteau, Huveaune, Toulon PR and Hyères presented significantly higher values than control mussels. Generally, lysosomal volume to surface ratio (S/V_{LYS}) (Fig. 2C) was highest in mussels from Corsica. Regarding mussels from different stations in the West Rhône region, individuals from Montpellier and Rhône presented significantly higher S/V_{LYS} values when compared to mussels from Aude. When comparing sampling sites in the East Rhône region, mussels sampled in Bregançon presented significantly higher S/V_{LYS} values than mussels from Toulon PR and Huveaune. Mussels from Aude and Hérault displayed significantly lower S/V_{LYS} values when compared to control mussels. Lysosomal numeric density (Nv_{LYS}) was significantly lower in mussels from Corsica compared to the values from other two regions (Fig. 2D). Individuals from Hérault, Ponteau, Huveaune, Toulon PR and Hyères presented significantly higher Nv_{LYS} values than the control mussels.

3.2.3. Intracellular neutral lipid accumulation

Regional neutral lipid volume density values (Vv_{NL}) were lowest in mussels from Corsica (Fig. 3). Mussels from the West Rhône region showed the highest Vv_{NL} values, while mussels from the East Rhône region presented intermediate general values. When comparing mussels sampled in different sites in West Rhône, mussels from Aude presented significantly higher Vv_{NL} values than mussels sampled in Rhône. Among the mussels sampled in the East Rhône region, individuals sampled in Toulon STP presented significantly lower values when compared to mussels from Ponteau, Huveaune and Toulon PR. Mussels from Bregançon and Emb. Du Var presented significantly lower values compared to mussels from Huveaune and Toulon PR. Individuals sampled in Aude, Hérault, Montpellier, Rhone, Ponteau, Huveaune, Toulon PR and Hyères displayed significantly higher Vv_{NL} values compared to control mussels.

3.3. Gamete developmental stages

The predominant gamete developmental stage found in the present work was the post-spawning phase (Fig. 4), which was close or above 60 % in the majority of the groups. Exceptions were found in mussels from Montpellier and control mussels, as they presented a higher percentage of mature gametes (50 % and 17 % respectively) and ongoing spawning phase (8 % and 67 % respectively).

3.4. Tissue-level biomarkers

Atrophy levels of digestive alveoli epithelium (Fig. 5A) were only significantly higher in mussels from Corsica when compared to the rest of the regions. Connective tissue index values (Fig. 5B) were overall higher in mussels from Corsica compared to general values in the other regions. Mussels from Bastia N STP, Bastia S STP, Golu S, Ajaccio STP and Ajaccio Gravone STP presented significantly higher connective tissue index values when compared to mussels from the control group. ADG cell index (Fig. 5C) values were in general significantly lower in mussels from Corsica when compared to general values from West Rhône and East Rhône regions. When comparing ADG indexes in mussels collected in different sites in the East Rhône region, individuals from Huveaune presented significantly higher values when compared to mussels from Toulon STP and Bregançon. Mussels sampled in Aude, Hérault, Rhone, Ponteau, Huveaune, Marseille STP, Toulon PR and Hyères presented significantly higher ADG values than mussels from the control group.

3.5. Histopathological lesions and parasites

Among the most remarkable histopathological findings (Table 2), the presence of turbellarian parasites in the gill lamellae was ubiquitous, prevalence ranging from 8 % to 33 % in all the groups. Intracellular ciliates located in digestive cells were found in a prevalence that ranged from 8 % to 42 %, except in mussels from Emb. Du Var, Hyéres, Toulon STP, Rhône and control mussels. Brown cell infiltration in digestive gland tissues was found more prevalently (25–58 %) in Emb. Du Var, Bregançon, Hyères, Toulon PR, Marseille STP, Rhône, Hérault, Ajaccio STP and the control mussels. Brown cell infiltration in digestive gland tissues was found less prevalently (>0 %- < 25 %) in Toulon STP, Ponteau, Aude, Montpellier, Ajaccio Gravone STP, Golu S, Bastia S STP and Bastia N STP. Atresia was found in the highest prevalence in the control group (75 %). Haemocytic infiltration prevalence was highest in Hérault (42 %) while in Emb. Du Var, Bregançon, Hyéres, Toulon PR, Toulon STP, Marseille STP, Huveaune, Rhone, Aude, Montpellier, Golu S, Bastia S STP, Bastia N STP and the control group prevalence ranged between 8 % and 25 %.

4. Discussion

The biological data reported in the present study provides critical information for the adequate ecosystem health status assessment using mussels as sentinel species in the Mediterranean Sea, and complement the analytical determination of contaminants presented in Briand et al.,

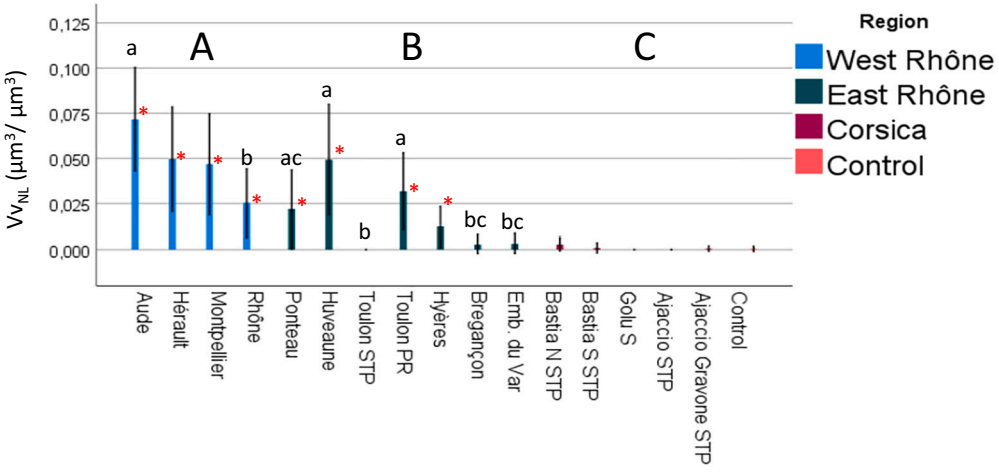

Fig. 3. Mean neutral lipid volume density (Vv_{NL}, n = 10). Large letters mean statistical differences between regions, small letters indicate significant differences between sampling sites in the same region and asterisks indicate differences when compared to the control group ($p < 0.05$).

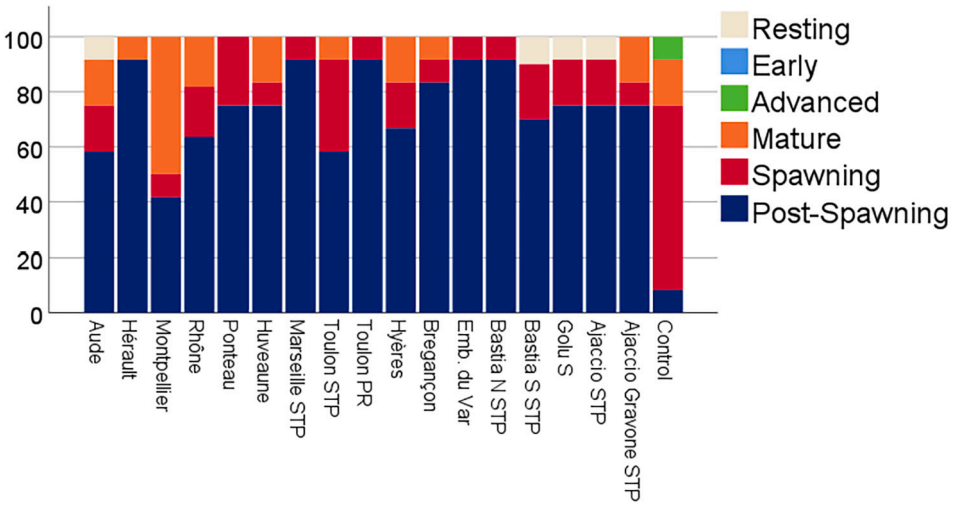

Fig. 4. Gamete developmental stages (%, $n = 12$).

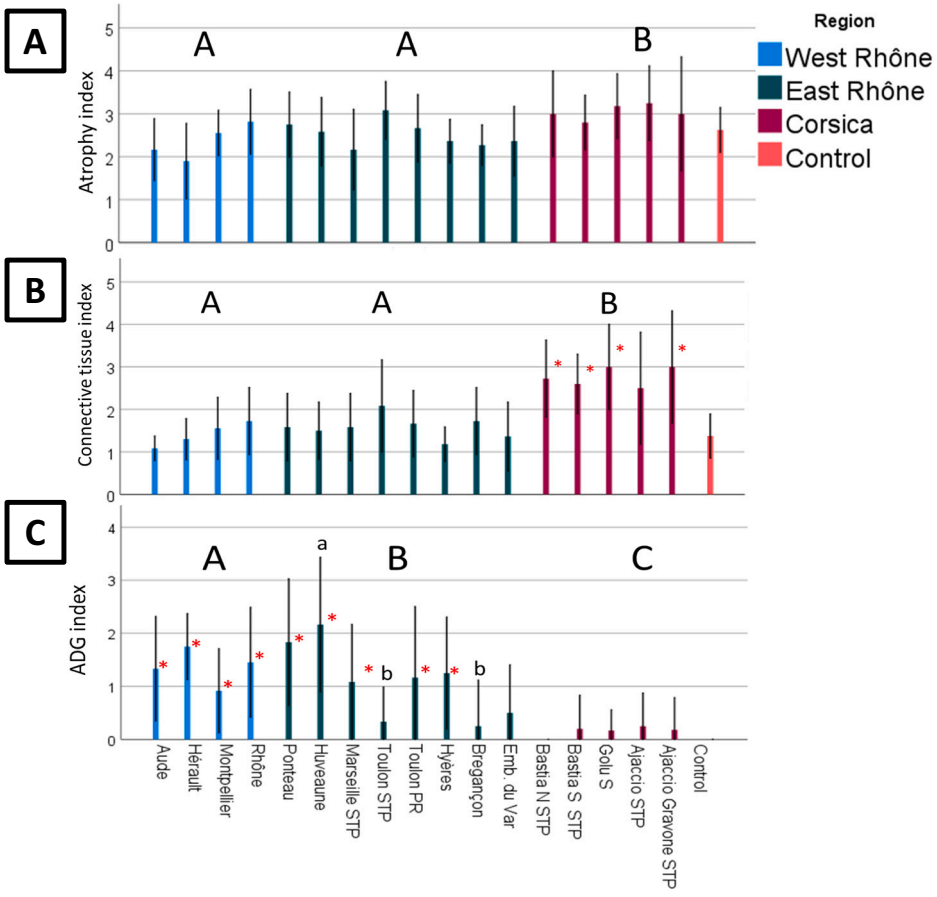

Fig. 5. A: Mean atrophy index (n = 12). B: Mean connective tissue index (n = 12). C: Mean adipogranular (ADG) cell index (n = 12). Large letters mean statistical differences between regions, small letters indicate significant differences between sampling sites in the same region and asterisks indicate differences when compared to the control group ($p < 0.05$).

2023. The potential effects of a mild environmental contamination, of natural factors (i.e. trophic condition) and of the caging strategy on the biological responses assessed here are discussed below. The conclusions reached could be of critical importance for the future establishment of active biomonitoring campaigns in the geographical area.

In the present study, the Chl-*a* concentration, $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios and C/N ratio were used as proxies of primary production and estimation of food sources. Overall, these parameters allowed identification of three main regions in the studied area differentiated by their nutrient status (Harmelin-Vivien et al., 2008, 2010). The first region was West Rhône, characterised by a marked land-derived influence from the Rhône River with mesotrophic waters (low $\delta^{13}C$, high $\delta^{15}N$ and higher Chl-*a* concentration). The Rhone River is a major source of organic matter for the West Rhône region as it creates a North Mediterranean

coastal current toward the Catalan coast (Dumas et al., 2015; Many et al., 2021). In comparison, the Corsican region was characterised by oligotrophic waters under oceanic influence (lower Chl-*a* concentration, high $\delta^{13}C$ and low $\delta^{15}N$; Sarà et al., 1998) and the East Rhône region showed an intermediate status with an oceanic influence and a secondary but multiple land-derived influence (e.g. from the Huveaune and Var). Overall, these parameters highlighted the existence of different food sources and levels of biological productivity between regions as also reflected by the higher C/N ratio detected in the West Rhône region than Corsica (Many et al., 2021). Thus, although mussels used for the present study originated from a same initial batch, differences in food sources between regions may generate changes in mussels' general status as observed in CI and FC (Table 1). In the case of the West Rhône region, the higher biological productivity of the area (Many et al., 2021) may affect individual's growth and contaminant bioaccumulation with a potential relative reduction of metal levels or an accumulation of organic compounds in lipid tissues (e.g. PCBs, DDTs) (Briand et al., 2023). Indeed, several studies have reported the effects of primary production (as a proxy of food availability) at different levels of biological complexity in *Mytilus* sp. (Andral et al., 2011; Farcy et al., 2013; Benito et al., 2019), which makes the food availability and nutritive status of the mussels important confounding factors for biomarker interpretation.

The reason behind using mussels from a same origin and the same size-range was to obtain a homogeneous population of individuals in similar initial health conditions when transplanted (Andral et al., 2004). This is particularly important regarding gametogenesis, as the reproductive cycle strongly influences the general condition of organisms and their capacity to respond to environmental stressors (Blanco-Rayón et al., 2020; Benito et al., 2019). In the present study, >60 % of individuals collected from the 17 sites were identified in post-spawning period. This is coherent with the gametogenic conditions detected for the control group, where most individuals were in spawning period before transplantation. Therefore, the histological examination of gonad samples confirms the progression of the reproductive cycle during this three-month period and proves homogeneity between batches of mussels, with the exception of mussels from Montpellier. Indeed, 50 % of mussels from this site presented mature gametes, a gametogenic difference that could condition the biological stress responses in comparison to the other sampling sites.

Although the mortality rates were relatively low in most of the cages, it is noteworthy that 23 % of the mussels from Bastia N STP were retrieved dead from the cages, a percentage that could be indicative of important distress. Indeed, animals sampled from this site presented a high connective tissue index and a low ADG index, together with the lowest CI and the second lowest FC, all indicating a very poor health status. Furthermore, mussels from this sampling site presented the highest prevalence of IC parasites (42 %). Briand et al. (2023) reported among the highest Cd (2.08 mg/kg dw) and Pb (2.12 mg/kg dw) concentrations compared to the rest of mussels sampled in the Corsica region. Those levels of Cd are comparable to values reported in a previous caging experiment in the Mediterranean Sea (Casas et al., 2008). The moderate chemical stress of Bastia N STP, together with the low nutritive condition of the animals, and the stress generated by the parasites, might have caused such mortality rates.

Lysosomal parameters presented clear differences among regions. Highest general LP values were registered in Corsica, together with lower Vv_{LYS} values (smaller and less lysosomes) and very low Vv_{NL} levels. Accordingly, intracellular lipid accumulation varied together with the trophic condition of each region. It has been described that among other natural factors, digestive activity influences lysosomal biomarkers in a significant manner (Izagirre et al., 2008) and the fact that smaller lysosomes might present higher LMS values (Izagirre and Marigómez, 2009) is coherent with the present results.

Table 2
Prevalence (%) of Turbellarian commensals, Intracellular Ciliates (IC), brown cell infiltration, atresia of oocytes and haemocytic infiltration identified in each station (n = 12). dg: digestive gland.

	Turbellaria	IC (dg)	Brown Cell Inf. (dg)	Atresia	Haemocytic inf.
Aude	17	17	8	8	17
Hérault	25	17	25	0	42
Montpellier	8	8	17	0	8
Rhône	17	0	25	0	25
Ponteau	33	8	8	0	0
Huveaune	25	33	0	0	17
Marseille STP	8	33	33	8	8
Toulon STP	8	0	5	8	17
Toulon PR	8	17	33	8	25
Hyères	8	0	25	0	8
Bregançon	8	17	25	8	25
Emb. du Var	8	0	33	8	8
Bastia N STP	17	42	17	0	17
Bastia S STP	8	8	8	0	17
Golu S	25	17	17	0	25
Ajaccio STP	17	25	58	0	0
Ajaccio Gravone STP	17	25	17	0	0
Control	8	0	42	75	25

Table 1
Values of the parameters obtained during the sampling campaign in each caging site: Chl-*a* concentration in water column (percentile 90, P90, μg/L), isotopic ratios in soft tissues of mussels (δ13C and δ15N, ‰, *n* = 15–25), C/N ratio in soft tissues of mussels (n = 15–25), mortality rates (M, %), mean condition index (CI, n = 15–25) and fat content (FC, %, n = 15–25). Asterisks indicate significant intraregional differences ($p < 0.05$).

Region	Station	Chl-a	δ13C	δ15N	C/N	M	CI	FC
West Rhône	Aude	1.64	−22.30	5.02	5.79	7.5	0.141	1.40
	Hérault	1.57	−21.98	4.88	5.56	16	0.132	1.04
	Montpellier	1.14	−21.77	4.18	5.20	9.2	0.112	1.19
	Rhône	2.13*	−21.26	4.87	4.87	8.8	0.100	1.12
East Rhône	Ponteau	1.40	−21.08	4.18	4.40	16	0.086	1.12
	Huveaune	1.21	−21.15	5.05	5.03	13	0.140*	1.22
	Marseille STP	1.41	−21.62	3.57	4.46	9.9	0.111	1.06
	Toulon STP	0.45	−21.26	3.86	3.95	11	0.065	0.92
	Toulon PR	2.03*	−21.81	5.20	5.29	4.4*	0.122	1.42*
	Hyères	0.91	−21.84	3.70	4.33	12	0.071	1.00
	Bregançon	0.35	−21.34	3.67	4.06	16	0.067	0.77
	Emb. du Var	1.94*	−19.34*	4.58	4.61	9	0.073	0.88
Corsica	Bastia N STP	0.45	−21.11	3.85	4.07	23*	0.051	0.62
	Bastia S STP	0.38	−19.90	3.67	4.18	12	0.070*	0.60
	Golu S	0.73	−21.06	3.52	3.94	10	0.052	0.71
	Ajaccio STP	0.65	−21.21	4.00	3.17	12	0.053	0.76
	Ajaccio Gravone STP	1.69*	−21.21	3.83	2.86	9.5	0.056	0.85
	Control	ND	−21.45	2.59	4.76	ND	0.076	1.35

Regarding intraregional differences in LMS, among the sampling sites located in West Rhône, Hérault presented significantly lower LP values compared to mussels from Aude and Rhône. Mussels from Hérault did not display worrying chemical burden (Briand et al., 2023) which could have been caused by the expulsion of the lipophilic pollutants during spawning together with the gametes as mussels from the same site displayed the most advanced gametogenic status in the region. Moreover, the histopathological analysis indicated the highest haemocytic infiltration prevalence in the same group. These kind of inflammatory reactions are often related to the presence of pollutants, parasitic infection and spawning stress (Pieters et al., 1980; Garmendia et al., 2011). Among sampling sites in Corsica, mussels from Bastia S STP presented significantly lower LMS values when compared to the ones sampled in Ajaccio Gravone STP. Although no apparent cause was detected in the present study, both groups presented mean values of 20 min or above, which is indicative of good health status in mussels (Marigómez et al., 2013a; Martínez-Gómez et al., 2015).

The values obtained for the three LSC parameters are comparable to the results previously reported in Mediterranean Sea mussels (Zorita et al., 2007) and stay below the threshold considered for good environmental conditions (Marigómez et al., 2013a). For instance, Vv_{LYS} values in mussels from reference sites commonly range from 0.002 to 0.004 $\mu m^3/\ \mu m^3$ (Izagirre and Marigómez, 2009; Marigómez et al., 2013a). Therefore, the stress levels reflected in this endpoint are probably of mild significance. Nevertheless, intraregional differences were found when comparing sampling sites in West and East Rhône. In East Rhône, mussels from Huveaune and Toulon PR presented the highest Vv_{LYS} values, which could be related to the moderate concentration of total PCBs in the first and the high Hg, total PAHs and total PCBs and the moderate Pb and Hg concentrations in the later for the same population of mussels (Briand et al., 2023). This is also coherent with the lower S/V_{LYS} values detected in the same sites when compared to mussels from Bregançon, which is a site with low chemical concentrations (Briand et al., 2023). Overall, S/V_{LYS} values recorded in the present study were higher (smaller lysosomes) than the reference value reported by Zorita et al. (2007) in native mussels from the Mediterranean coast. This could be an alteration caused by the effect of caging (Marigómez et al., 2013b), or an improvement of the general health status in Mediterranean Sea. Regarding Vv_{NL}, values reported in the present study are not high enough to demonstrate a clear lipid accumulation related to organic contaminants (Marigómez and Baybay-Villacorta, 2003). Instead in the West Rhône region, higher Vv_{NL} values detected in Aude compared to Rhône are concordant with the higher CI and FC values displayed by mussels sampled in the former. In the East Rhône region, mussels from Huveaune and Toulon PR presented significantly higher Vv_{NL} values when compared to mussels from Toulon STP, Bregançon and Emb. du Var, which is concordant with higher Vv_{LYS} values in Huveaune and Toulon PR. One of the advantages of active monitoring is the possibility to have a complete characterization of the mussels used. Among the groups showing significantly different lysosomal membrane stability, some display lower LP values than the control group, which could be explained by the effect of contaminants (Ponteau, Huveaune), as reported by Briand et al. (2023), by physiological stress, such as ongoing gametogenesis (Montpellier), or additional pathological conditions (Hérault). In the case of mussels sampled in Bregançon and Toulon STP there is no clear cause for the low LP values, although both sites presented low Chl-*a* concentrations compared to other sites in the same region and could reflect an early lysosomal response to starvation, which is coherent with the lower Vv_{NL} values. Mussels from Ajaccio STP and Ajaccio Gravone STP displayed significantly higher LP compared to the control mussels, which as discussed before could be a consequence of the smaller and more stable lysosomes showing a reduced digestive activity. Mussels from Aude, Hérault, Montpellier, Ponteau, Huveaune, Toulon PR and Hyères presented higher Vv_{LYS} and Nv_{LYS} (also the first two sites presented lower S/V_{LYS} values) when compared to control mussels potentially indicating an enlarged *endo*-lysosomal system, which is concordant with the general trophic conditions discussed in the intraregional differences part. The significantly higher Vv_{NL} values displayed by the caged animals when compared to control mussels are probably related to better trophic condition in those sites together with the lack of reproductive activity. Similar conclusions regarding trophic conditions and neutral lipid accumulation in Icelandic mussels and clams have been reported by Nahrgang et al. (2013).

In general, regarding tissue-level biomarkers, mussels from Corsica presented higher values of atrophy and connective tissue index compared to the other regions. This is concordant with the general CI and FC, indicating that lower primary production and subsequent lower reserve material storage might cause a general stress signal in tissue-level biomarkers, as it has been described before in mussels under certain environmental conditions (Benito et al., 2019). Mussels from Corsica also presented the lowest general ADG index which is concordant with the high atrophy and connective tissue values and the low CI and FC measurements, as ADG index works as a proxy of energy storage materials in the mantle (Bignell et al., 2011). It can be concluded that regarding tissue-level biomarkers, environmental variables such as primary production are influencing stress responses to pollutants.

As stated before, the only intraregional differences regarding tissue-level biomarkers were found regarding ADG index in mussels from East Rhône, since mussels from Huveaune presented significantly higher values than mussels from Toulon STP and Bregançon. The high ADG index values in Huveaune could be explained by the relatively high CI, FC and Chl-*a* values. Mussels from Toulon STP presented one of the lowest CI values in the region, together with low Chl-*a* concentrations. Although the Chl-*a* concentrations measured in Emb. du Var were relatively high, mussels caged in this site presented relatively low CI and FC, which could have been caused by the moderately high As and CR and high Mn concentrations in soft tissues (Briand et al., 2023).

Connective tissue index was found to be different between control musseIs and mussels from every site in Corsica region. As discussed before, the high connective tissue index recorded in Corsica might be caused by poor dietary conditions, which were prolonged until July, while the unfavourable trophic conditions in the control mussels only lasted until March. Differences in ADG index were detected between control mussels and mussels caged in the West Rhône region, consistent with the association between favourable trophic conditions and a high ADG index.

As far as the authors are aware, there is no comparable data regarding the tissue-level biomarkers used in the present work. However, endpoints applied herein could result of great utility for future works as reference values, as concluded by other studies that applied similar set of tissue-level biomarkers on wild mussels in other regions (Benito et al., 2019, 2023). In addition, previous works (Marigómez et al., 2013b) described similar tissue-level responses toward contamination in native and caged mussels, which could mean that tissue-level biomarkers are less prone to be biased by the effect of caging in active monitoring campaigns.

Histopathological analysis confirmed the presence of certain parasites and alterations that could have an impact on the health status of caged mussels. A good example is the turbellarian commensal found in almost all the groups. The higher prevalence found in caged mussels when compared to control mussels could be related to the season as this commensal is more prevalent in summer (Crespo-González et al., 2010), when caged mussels were sampled, while control mussels were sampled earlier, in March. Although no major host reaction was observed in most cases in the present study, some mussels presented associated haemocytic infiltration, which indicates that turbellarians might impinge some harmful effects on the host. This is coherent with previous studies that described haemocytic infiltration and necrosis of the gill tissues in relation to turbellarian presence (Robledo et al., 1994).

Similar IC parasites to the ones described in the current work were previously described as Multinucleate Parasite X (MPX) (Fichi et al., 2018). In the present study, the prevalence calculated for these parasites

was in line with data previously reported in Mediterranean mussels (Bhaby et al., 2013). The high variability detected in the infection prevalence herein could have been caused by the effect of pollutants (mussels from Huveaune, Marseille STP) that may affect the host immune system (Ayhan et al., 2021), potentially facilitating parasite infection. The high prevalence of IC in mussels from Corsica could be explained by the low nutritive status of the region, as environmental conditions may influence the hosts' infection prevalence (Bhaby et al., 2013).

Inflammatory responses such as brown cell infiltration in digestive gland tissues and haemocytic infiltration are closely related and can be caused by a different array of aetiological agents such as pollution, parasitism and starvation among others (Garmendia et al., 2011; Benito et al., 2022). In the present study, the highest brown cell infiltration prevalence in mussels from East Rhône (Marseille STP, Toulon PR and Emb. du Var) could be explained by the presence of pollutants and parasites, while the ones in Corsica could be related to bad nutritive status. The high prevalence of brown cell infiltration in control mussels could have been caused by ongoing gametogenesis. As it was stated previously, the highest haemocytic infiltration prevalence was found in Hérault, and although the cause is unclear, these kind of responses should be taken into account as an indicator of a compromised health status (Benito et al., 2022). Histological alterations associated with the reproductive cycle were found predominantly in control mussels, as it was the group presenting ripe gametes; in the case of atresia for instance, it is more probable to find affected oocytes when more mature gametes are present (Cuevas et al., 2015). This pathology is an indicator of ongoing autolysis and resorption processes during gametogenesis and can be induced when environmental conditions become unfavourable for spawning after gamete maturation (Smolarz et al., 2017).

Histopathological analysis is critical to detect possible effects of pathogens, parasites or pathologies that might alter general stress biomarkers as the ones used in the present work (Garmendia et al., 2011, Benito et al., 2022). Active biomonitoring programs performed by caging mussels could be helpful to avoid potential confounding effects of certain parasites present in native populations. In active monitoring studies, all caged animals display a similar “original” histopathological profile, which would only vary due to the effect of pollutants in the caging site or due to highly infective pathogens floating in the water column. However, although possible, the latter would not be very probable, as caged mussels would be usually at a great distance from potentially infective natural mussel populations. In addition, it has been demonstrated that natural mussel populations are more prone to suffer parasitic infections and subsequent histopathologies when they are collected from benthic areas with rich natural communities (Buck et al., 2005; Benito et al., 2022), giving a clear advantage to the active biomonitoring strategy in front of passive biomonitoring programs.

To summarize, the main aspects to take into account in the present active biomonitoring campaign were the high mortality rates registered in Bastia N STP; the generally low environmental contamination; the differential trophic condition of each region and the homogenous gametogenic status. Localized high mortality rates can be considered as a limitation for the use of this sampling site in further studies. The determination of environmental factors such as trophic conditions has been demonstrated to be critical in order to better interpret the effects provoked by pollutants that in the present work did not elicit evident responses, permitting the report of biomarker baseline values in the studied areas. The homogenous reproductive status presented by caged mussels in the present work allowed to discard the effect of differential reproductive conditions in biomarkers, confirming the adequacy of the experimental design. In conclusion, the present work reported valuable biological data of biomarkers and histopathological alterations widely used in biomonitoring campaigns, performing the health status assessment of a wide geographical area in the Western Mediterranean Sea through active biomonitoring approach. The general trophic condition of the three main regions was defined and its effect on general stress responses of mussels was clearly discriminated, allowing the detection of mild chemical impact on caged mussels and the report of novel data that could be used as reference values in the future.

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CRediT authorship contribution statement

Denis Benito: Writing – original draft, Visualization, Methodology, Investigation. **Marine Briand:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition, Conceptualization. **Olivier Herlory:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Urtzi Izagirre:** Writing – review & editing, Visualization, Validation, Investigation, Conceptualization. **Marc Bouchoucha:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Tifanie Briaudeau:** Writing – review & editing, Methodology, Investigation, 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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