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Integrated assessment of biological responses to pollution in wild mussels (*Mytilus edulis*) from subarctic and arctic areas in the Norwegian sea[☆]

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ABSTRACT

North Atlantic and Arctic Oceans contain large amount of undiscovered oil and gas reserves. Therefore threat of oil spills and its hazardous ecological consequences are of great importance to the marine environment. Although mussels (*Mytilus* sp.) respond clearly to contaminants, biomarkers have shown variability linked to biological and environmental changes. In order to help avoiding misinterpretation of biological responses the aim of this study was to reveal the effect of natural variability in the responsiveness to pollution of a battery of cell and tissue-level biomarkers in mussels. Mussels were collected in relatively non-impacted and potentially impacted sites at ports and the vicinity of a waste water treatment plant in Trondheim and Tromsø in autumn of 2016. Although the battery of biomarkers used herein proved to be useful to discriminate impacted and non-impacted mussel populations, some confounding factors altering the biological responses were identified. Geographical/latitudinal factors seemed to be critical regarding the reproductive cycle, reserve material storage and the prevalence of parasites such as *Gymnophallus* cf. *Bursicola* trematodes. Mussels from the reference site in Tromsø displayed general stress responses at different levels, which could be influenced by the pathogenic effect of the *Gymnophallus* cf. *Bursicola* trematode and by a more advanced gametogenic developmental stage compared to the mussels from Trondheim, which could lead to misinterpretation of the reasons behind the measured stress levels in those mussels. Despite these confounding effects, the use of integrative tools such as IBR index helped to discriminate mussel populations from chemically impacted and non-impacted sites. Overall, this work serves as an anchor point both as a reference of the baseline level values of the analyzed endpoints in the studied geographical area and time of the year, and as an indication of the potential extent of the environmental confounding factors in monitoring programs causing stress on the analyzed mussel populations.

1. Introduction

Marine ecosystems are at risk of being affected by elevated concentrations of contaminants released from anthropogenic sources. The concentrations of PAHs, PCBs and metals in European coastal waters are mostly decreasing, but the amount of data is scarce in some areas, including the Norwegian Sea (EEA, 2018). On the other hand, North Atlantic and Arctic Oceans contain noteworthy amount of both discovered and undiscovered oil and gas reserves (Gautier et al., 2009). Due to the rapid warming and substantial loss of sea ice in the Arctic, the travel between Europe and Asia through the Northern Sea Route, mainly conducted by dry cargo ships, oil tankers, and liquefied natural gas

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carriers, increased over the last decades (Chen et al., 2022). Under these circumstances, the possibility of an oil spill accident in this area may turn out to be real and their potential hazardous ecological and socio-economic consequences need to be studied. It is to be taken into account that Arctic ecosystems are highly vulnerable to oil spills due to their unique environmental conditions (low temperature of seawater and, more remarkably, the presence of ice-cover) and remoteness as these features could not only modify the chemical composition of the spilled products and intensify the toxicity to marine biota (Word, 2014; Nordam et al., 2017) but also hamper the oil spill responses and subsequent cleaning-up operations.

Mussels (*Mytilus* sp.) are widely used as sentinel species of marine pollution as they are ubiquitous, sessile, filter feeding organisms placed on a low trophic level. They accumulate contaminants in their tissues and allow the monitoring of the bioavailable fraction of contaminants over time along a wide geographical distribution (Viarengo et al., 2007; Bellas et al., 2014; Cuevas et al., 2015; Beyer et al., 2017). Even though they respond to contaminants, biomarkers have shown a variability due to biological and environmental changes and temporal fluctuations occurring in nature (Depledge, 2009; Fernández et al., 2010; Nahrgang et al., 2013; Beyer et al., 2017; Benito et al., 2019). Throughout their lifetime, mussels experience biochemical, metabolic and/or physiological changes which might act as confounding factors altering the organisms' ability to respond to the presence of pollutants. Consequently, there is a need for deeper understanding of the effect exerted by biological cycles on them and the baseline levels of the biomarkers in question in order to correctly interpret biomarker responsiveness (Nahrgang et al., 2013; Beyer et al., 2017; Storhaug et al., 2019).

The blue mussel (*Mytilus edulis*) has a wide distribution in the boreo-temperate region, in the North-Pacific, North- and Mid-Atlantic up to the Arctic Ocean (Kijewski et al., 2011). Their growth and reproduction largely depends on temperature and food availability (Thorarinsdóttir and Gunnarsson, 2003; Berge et al., 2005; Beyer et al., 2017; Storhaug et al., 2019). Consequently, there can be a misinterpretation of biomarker responses due to the potential confounding factors related to mussels' reproductive condition, the responsiveness of biomarkers against pollution being compromised. Hence, it is necessary to decipher how the natural variability of these confounding factors can affect biomarker responses (Bignell et al., 2008; Cuevas et al., 2015; Bellas et al., 2014; Beyer et al., 2017).

This study aims to reveal the effect of natural variability on the responsiveness of a battery of selected biochemical, cell and tissue-level biomarkers in mussels collected both, in relatively non-impacted and potentially impacted sites located in the Norwegian coast.

2. Material and methods

2.1. Sampling

The collection of mussels (*Mytilus edulis*) was performed in three sampling sites in Trondheimsfjord and one in Åjford, which were clustered as the Trondheim area sites. These included an allegedly non-impacted site used as a reference site in Rissa (63.561753, 9.899776) on the October 18, 2016, a port in Trondheim (63.442692, 10.425494) on the October 17, 2016 and a rocky beach in the vicinity of a wastewater treatment plant (WWTP) effluent in Trondheim (63.444867, 10.341331) on the October 19, 2016. Farmed mussels from a long-line in Åjford, Trøndelag (63.940837, 10.160624) were also collected at a mussel depuration facility in Rissa on the October 18, 2016. On the other hand, the two sampling sites in Tromsø included a rocky beach in an allegedly non-impacted site used as a reference site (69.642089, 18.94639) and a port (69.654177, 18.968459). Mussels in both sampling sites were collected on the October 20, 2016.

Mussels, sized 3.5–4.5 cm long (except in Trondheim port that were 2–4.5 cm long) were sampled from the first meter of the lower intertidal zone, taken to the laboratory in air at ambient temperature and dissection was carried out immediately upon arrival. In each dissection, transversal slices including mantle, gills and digestive gland were performed in 20 mussels for histopathological analysis and tissue-level biomarkers. Moreover, 20 mussel digestive glands were dissected out and snap frozen in liquid nitrogen, then stored for biochemical and cellular biomarkers. Additional samples of whole mussels were frozen for chemical analysis of soft tissues.

2.2. Sample processing

The transversal slices were processed as indicated in Benito et al. (2022). Detailed information is available as supplementary material.

2.3. Chemical analysis of polycyclic aromatic hydrocarbons (PAHs) and trace metals

Chemical analysis of mussel tissues was carried out in duplicate following the method described by Navarro et al. (2009). Regarding the PAHs, from the list of 16 priority ones we measured 12, leaving aside the ones with higher volatility (naphthalene, acenaphthylene and acenaphtene). Regarding the metals total concentrations of V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Hg, and Pb were measured by ICP-MS after the digestion in a microwave oven as described by Bartolomé et al. (2010). As described by Mali et al. (2022), the measured PAHs were identified by the ratios of light (2 and 3 aromatic rings) and heavy (4 or more aromatic rings) and the ratios of some specific isomers. Detailed information is available as supplementary material (Table 1).

2.4. Biochemical biomarkers

Digestive gland tissues were stored in liquid nitrogen and transported to University of Iceland's Research Centre in Sudurnes where the biochemical analysis was performed, always maintaining a maximum temperature of −80 °C. Catalase activity (CAT) was assessed together with Glutathione-s-Transferase and Glutathione Peroxidase activities and Malondialdehyde levels, the data from the last 3 endpoints are included as supplementary materials (Table 2). Detailed methodological information is available as supplementary material.

2.5. Adipogranular cell index

As an indicator of the reserve energy material in the mantle, the adipogranular (ADG) cell index can give a hint of the metabolic strategy related to reproduction and seasonal bioenergetic changes (Bignell et al., 2008; Benito et al., 2019). Detailed information is available as supplementary material.

2.6. Gamete development

Gamete developmental stages were determined in histological slides (n = 10) as described by Ortiz-Zarragoitia et al. (2011) and Benito et al. (2019). Detailed information is available as supplementary material.

2.7. Cellular biomarkers

Lysosomal membrane stability (LMS) test and lysosomal structural changes (LSC) are general stress biomarkers (Marigómez et al., 2006; Izagirre et al., 2008; Benito et al., 2019). The determination of LMS was based on the time of acid labilisation treatment required to produce the maximum staining intensity according to UNEP/RAMOGE (1999) after the demonstration of hexosaminidase (Hex) activity in digestive cell lysosomes (Marigómez et al., 2006; Izagirre et al., 2008; Benito et al., 2019). The histochemical activity of β-glucuronidase was demonstrated in unfixed cryotome sections (10 digestive glands per group) as described by Moore (1976). Detailed information is available as supplementary material.

Table 1
Prevalence (%) of *Gymnophallus* and *Renicola* sp. Trematodes, *Mytilicola* sp., brown cell infiltration in digestive gland and gonad, atresia of oocytes, granulocytoma and haemocytic infiltration. DG: digestive gland, G: gonad.

		Gymnophallus	Renicola sp.	Mytilicola sp.	Brown cell (DG)	Brown Cell (G)	Atresia	Granulocytoma	Haemocytic inf.
Trondheim	Reference	0	0	5.56	0	0	5.56	0	16.67
	Farm	0	0	0	0	5	0	0	15
	Polluted	0	0	5.26	0	0	5.26	0	47.37
Tromsø	WWTP	0	45	0	10	0	30	5	40
	Reference	9.53	14.29	0	0	0	45	25	50
	Polluted	15	0	0	0	25	35	10	50

Intracellular neutral lipid (NL) accumulation in digestive gland has been related to organic xenobiotic exposure, non-specific stress and nutritional status (Cancio et al., 1999; Marigómez and Baybay-Villacorta, 2003; Shaw et al., 2011; Benito et al., 2019). Detailed information is available as supplementary material.

2.8. *Tissue-level biomarkers and histopathology*

Paraffin sections (5 μm) were stained with haematoxylin-eosin (H/E) to analyze the integrity of the digestive gland, gonad and gills. Vv_{BAS} is a general stress biomarker that measures the relative increase of basophilic cells under stress conditions in the digestive alveoli of molluscs (Zorita et al., 2006; Rementeria et al., 2016; Benito et al., 2017; Benito et al., 2019). A high connective to diverticula (CTD) ratio indicates a loss of the integrity of the digestive gland of molluscs, and it can be caused by a chemical stressor or by an insufficient nutritional status (Múgica et al., 2015; Benito et al., 2017; Benito et al., 2019).

Both, Vv_{BAS} and CTD, were quantified ($n = 10$) by means of stereology as an indication of whether changes in (a) cell-type composition, (b) in mean digestive epithelium thickness and (c) in the relative amount of connective tissue occurred or not (Benito et al., 2019). Detailed information is available as supplementary material.

Epithelial thinning of the digestive alveoli of mussels measured as atrophy index can be indicative of general stress (Kim et al., 2006; Garmendia et al., 2011; Benito et al., 2019). The severity of the atrophy index of the digestive alveoli was rated using a numerical grading from 0 to 4 ($n = 20$) as described by Kim et al. (2006) and Benito et al. (2019). Detailed information is available as supplementary material.

For histopathological analyses, slides of 20 mussels per sampling campaign were examined individually under the light microscope using 10 × , 20 × or 40 × objective lenses. Detailed information is available as supplementary material.

2.9. *Integrated biological response (IBR) index*

The IBR index was based on the integration of five biological responses from biochemical to tissue levels (CAT, LMS, Vv_{BAS}, CTD and ADG) to represent responses at different biological organization levels, according to Beliaeff and Burgeot (2002), Broeg and Lehtonen (2006) and Marigómez et al. (2013). Detailed information is available as supplementary material.

2.10. *Statistical analysis*

Statistical analysis was carried out with the aid of the SPSS/PC + statistical package V.24 (SPSS V.28, IBM SPSS Statistics, IBM Corp). For the quantitative data which passed normality test, one-way ANOVA and subsequent Duncan's post-hoc test for multiple comparisons between pairs of mean values was applied ($p < 0.05$). For the semiquantitative results obtained and the quantitative results which did not pass normality test, non-parametric Kruskal-Wallis ANOVA tests were carried out comparing the distribution using pairwise comparison ($p < 0.05$). Chemical burden Principal Component Analysis (PCA) plots were performed using Unscrambler X (v 10.5.1, CAMO, Norway).

3. Results

3.1. *Chemical burden in soft tissues*

In this case, the distribution of light and heavy PAHs in the soft tissues are plotted in Fig. 1 together with the Fluoranthene/(Fluoranthene + Pyrene) F/(F + P) ratio. The highest concentrations of PAHs were linked to both ports and the lowest to the Åfjord farm and reference sites. Additionally, higher concentrations of heavy PAHs (H-PAH, 4 or more aromatic rings) than the light ones (L-PAH, less than 4 aromatic rings) were observed in all the sites except in the farm in Åfjord.

In order to provide a broader view of the distribution of PAHs and trace metals (table in supplementary material), a principal component analysis (PCA) was carried out. As can be seen in the score and loadings projections shown in Fig. 2, the first two PCs explained up to 83% of the total variance, and the PC1-PC2 score plot revealed that animals from both ports were far away from the reference sites, the WWTP effluent and the farm. The specific patterns observed in the two ports can be explained according to the different nature of contaminants detected in those samples. In the case of Trondheim, the contamination was linked to the presence of metals such as V, Cu or Fe. While, on the contrary, in Tromsø, the contamination was linked to the different distribution pattern of Anthracene and Phenanthrene and a higher concentration in many metals (Fe, Mn, V and Cr).

3.2. *Biochemical responses*

Mussels from both reference sites contained significantly higher protein concentration in their digestive glands (Fig. 3A) compared to the ports. Catalase activity (Fig. 3B) did not differ between the stations. No correlation was observed between biochemical responses and metal content or PAH's. No biochemical analysis was performed in mussels from the Åfjord farm due to missing samples.

3.3. *Reproductive status*

ADG index (Fig. 4A) presented high interindividual variability with significant low levels detected only for mussels sampled in the port in Trondheim. Gamete developmental stages (Fig. 4B) showed differences between the mussels sampled in Trondheim and Tromsø. However, no relevant changes were detected when comparing mussels from reference sites and ports in the same locality except in the WWTP in Trondheim. Mussels from Trondheim (reference site and port) were mostly in early gametogenesis while the ones from the WWTP presented advanced gametogenesis. Mussels from Tromsø were predominantly in advanced gametogenesis.

3.4. *Cellular biomarkers*

Vv_{NL} (Fig. 5A) showed geographical differences, hence mussels form Trondheim exhibited significantly lower values than mussels from Tromsø. In addition, mussels from the port in Trondheim and Tromsø presented significantly lower values when compared to mussels from the reference sites in the same localities, while mussels from the WWTP and the Åfjord farm showed intermediate values. Mussels from reference

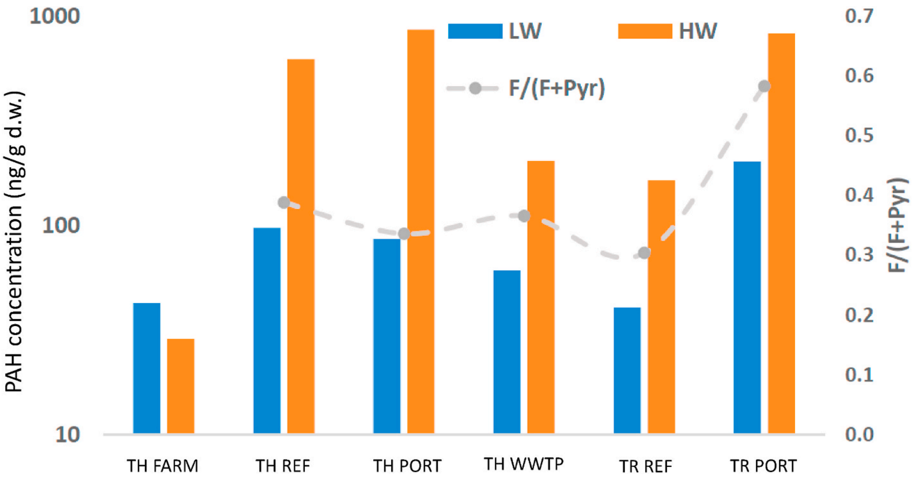

Fig. 1. Light (LW) and heavy (HM) molecular weight PAHs and the fluoranthene-pyrene F/(F + Pyr) ratios for the mussels tissues. TH: Trondheim, TR: Tromsø, FARM: mussel farm, REF: Reference site and WWTP: Wastewater treatment plant.

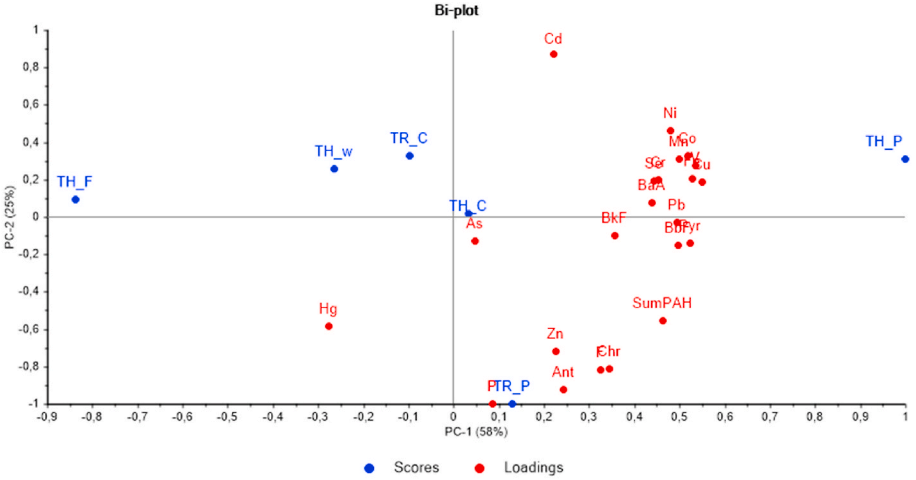

Fig. 2. Plot of the sample projection on the PC1-PC2 plane. These two PCs explain up to 83% of the total variation of the sampled groups caused by the concentration of pollutants in soft tissues of mussels and a slight clustering is observed in the reference sites (XX_C), the sample taken close the release of a WWTP (TH_W) and 2 of the three ports (XX_P). TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: port.

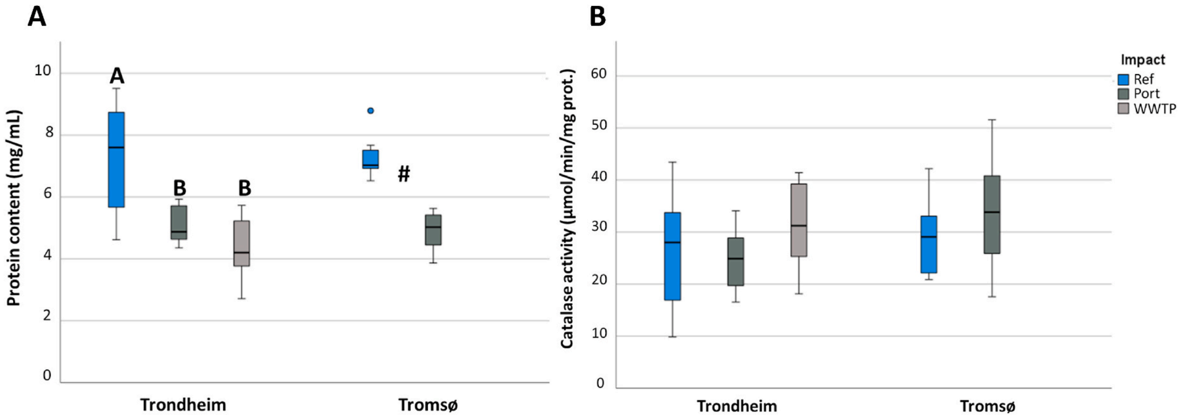

Fig. 3. Protein content (A) and catalase activity (B) in digestive gland of *M. edulis*. Different letters mean statistical differences between sampling sites in Trondheim. # indicates differences between both sites in Tromsø ($p < 0.05$). Ref, reference site; WWTP, Wastewater treatment plant; Port, port site.

sites and the Åfjord farm exhibited the highest LP values (Fig. 5B) when compared to ports in the same localities, ranging 15–18 min. Mussels from the WWTP in Trondheim displayed intermediate LP values.

Mussels from the port in Trondheim presented significantly lower Vv_{LYS} values (Fig. 6A) when compared to mussels from the reference site in the same locality, while mussels from the farm and the WWTP presented

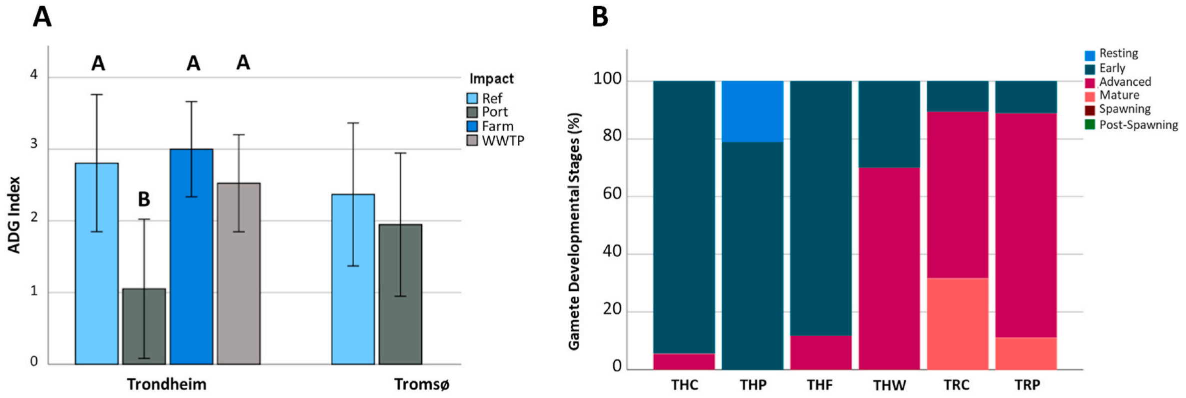

Fig. 4. A) Adipogranular cell (ADG) index in mantle tissue. Letters mean statistical differences between sampling sites in Trondheim ($p < 0.05$). Legend as in Fig. 3; Farm, mussel farm. B) Gamete developmental stages (%). TH: Trondheim, TR: Tromsø, C: Reference site, F: Farm, W: WWTP, P: Port.

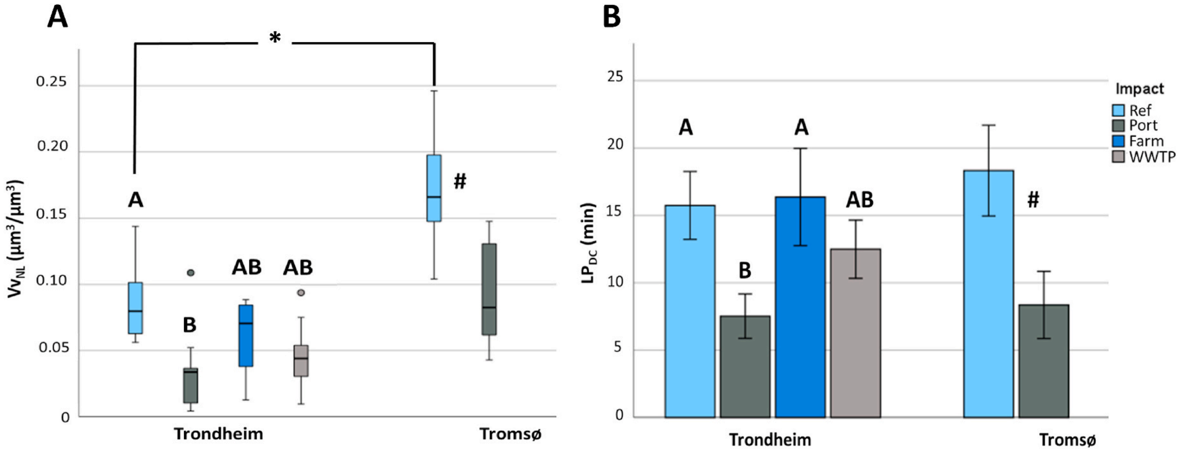

Fig. 5. A: Neutral lipid volume density (VvNL). B) Lysosomal membrane labilisation period (LP). Letters mean statistical differences between sampling sites in Trondheim. # indicates differences between both sites in Tromsø. * indicates statistical difference between the reference sites ($p < 0.05$). Legend as in Fig. 4A.

intermediate values. Significantly higher S/V_{LYS} (Fig. 6B) was measured in the mussels from the port in Trondheim and in the farm when compared to mussels from the reference site in Trondheim. Mussels from the port in Trondheim showed significantly higher NV_{LYS} values (Fig. 6C).

3.5. Tissue level biomarkers and histopathology

Vv_{BAS} levels (Fig. 6D) were significantly higher in mussels from the port in Trondheim when compared to mussels from the reference site in the same locality and the Åfjord farm. In addition, mussels from the WWTP in Trondheim displayed significantly higher Vv_{BAS} values than the ones from the farm. CTD ratio (Fig. 6E) was significantly lower in mussels from the reference site in Trondheim when compared to the results registered in the port and in the WWTP in the same locality. Atrophy index (Fig. 6F) was significantly higher in mussels from the port and the WWTP in Trondheim when compared with the ones from the reference site in the same locality. Mussels from the reference site in Tromsø showed significantly higher atrophy index values than the mussels from the reference site in Trondheim.

Parasitic burden (Table 1) was low in all sampling groups from Trondheim except in mussels from the WWTP, where the prevalence of *Renicola* sp. Trematodes was 45%. Mussels from the reference site in Tromsø presented a prevalence of 9.53% of *Gymnophallus* cf. *Bursicola* trematodes while the prevalence of *Renicola* sp. Parasites was 14.29%. Mussels from the port in Tromsø presented a prevalence of *Gymnophallus* cf. *Bursicola* trematodes of 15%.

Among the pathological alterations (Table 1) considered inflammatory reactions, brown cell infiltration in the digestive gland was only found in mussels from the WWTP in Trondheim (prevalence of 10%) and in mussels from the port in Tromsø (prevalence of 15%). Brown cell infiltration in gonad was present in mussels from the farm (5%). While in Tromsø, the 25% of the mussels from the port presented infiltration of brown cells in the gonad. Atresia of the oocytes showed low prevalence in all the mussels from the reference and the ports in Trondheim (below 13%). On the contrary, mussels in the WWTP in Trondheim presented a prevalence of 60%. In Tromsø, atresia of the oocytes was very prevalent in all groups ranging between 70% and 92%. The presence of granulocytomas was very low in Trondheim, only being found in 5% of the mussels from the WWTP. In mussels sampled in Tromsø, granulocytomas were more prevalent in both sampling sites, with mussels from the reference site showing a prevalence of 25% and mussels from the port showing a prevalence of 10%. The prevalence of haemocytic infiltrations was relatively low in the reference site and the farm in Trondheim while mussels from the WWTP presented a prevalence of 40%. In Tromsø mussels from the reference site showed a prevalence of 50%. Mussels sampled in the port presented a haemocytic infiltration prevalence of 50%.

3.6. IBR

The IBR index (Fig. 7A) displayed differential responses in mussels from each sampling site. Mussels from the reference site in Trondheim displayed the lowest stress levels in all the parameters, while mussels

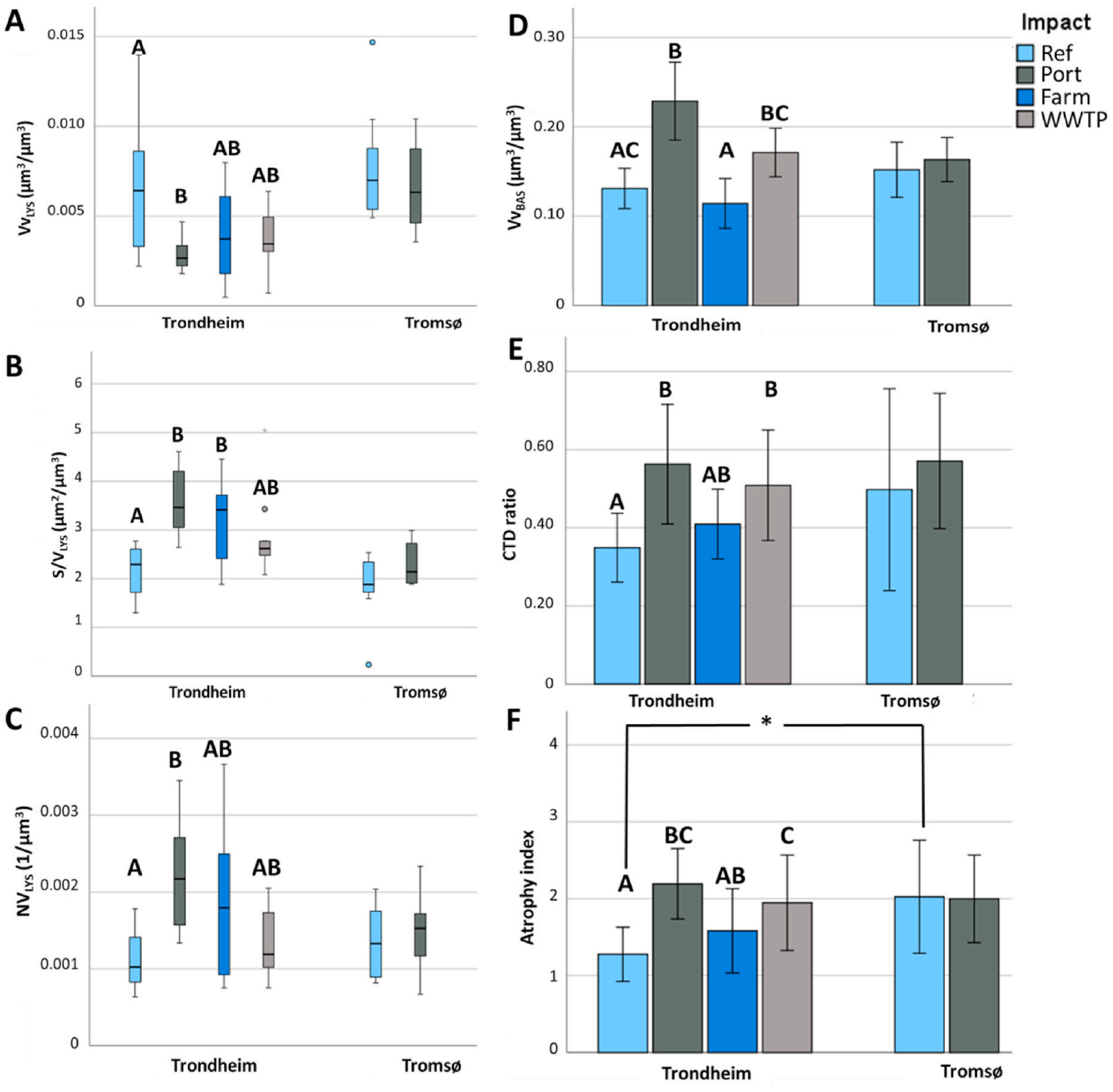

Fig. 6. A) lysosomal volume density (VvLYS); B) lysosomal surface/volume ratio (S/VLYS); C) lysosomal numeric density (NVLYS); D) basophilic cell volume density (VvBAS); E) Connective to diverticula (CTD) ratio and F) atrophy index in mussels sampled in Trondheim and Tromso. Letters mean statistical differences between sampling sites in Trondheim. * indicates statistical differences between both reference sites ($p < 0.05$). Legends as in Fig. 4.

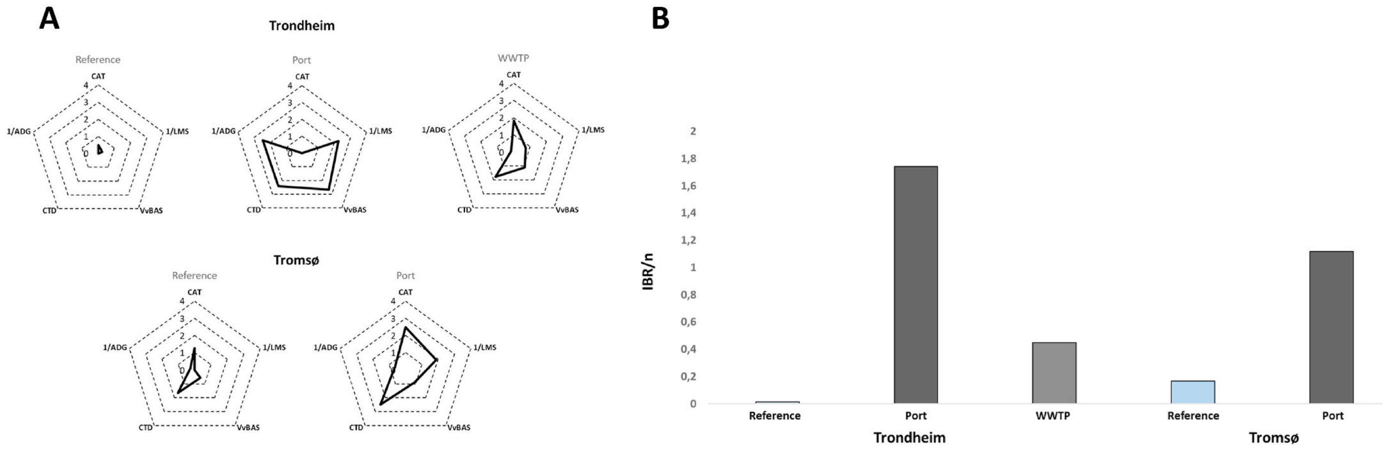

Fig. 7. Radar plots for biomarkers and the corresponding IBR/n index for Trondheim and Tromsø. LMS, lysosomal membrane stability test; VvBAS, volume density of basophilic cells; CTD, connective to digestive tissue ratio; ADG, adipogranular cell density.

from the port in the same locality presented high stress responses regarding LMS, Vv_{BAS}, CTD and ADG. Mussels from the WWTP presented relatively high stress responses in CAT and CTD. Mussels from the reference site in Tromsø presented mild stress responses mainly in CAT and CTD, while mussels from the port in the same locality displayed relatively high stress responses regarding CAT, LMS and CTD. The highest IBR/n (Fig. 7B) value was displayed by mussels from the Trondheim port followed by the port in Tromsø. Both reference sites presented lowest values while the WWTP in Trondheim presented slightly higher IBR/n value, although it was also relatively low. As there was no biochemical data for mussels from the farm in Trondheim, that group was not included in this index.

4. Discussion

As far as the authors are aware, the present study provides a first approach to reveal the responsiveness of a battery of selected biomarkers in mussels sampled in two localities (one in subarctic and the other at arctic latitudes) in the Norwegian Sea. In general terms, the final objective of the studies based on biomarkers is to assess the environmental health status of mussel populations, however, the knowledge of the influence of confounding factors (natural variability caused by latitude-related environmental and biological factors in the case of the present study) is crucial to correctly assess the impact caused by a potential environmental disaster (e.g. oil spill) in cold waters and to design efficient biomonitoring programs in latitudes with marked environmental differences.

The *a priori* considerations of reference and polluted sites are confirmed by chemical analyses, where polluted sites presented higher PAH and metal levels. According to Zhang et al. (2008), the low light-to-high PAH ratio ($\ll$1.0, except for mussels from the farm located in Åfjord) suggest a pyrogenic source. In addition to this, the ratio F/(F + P) is also used to indicate the source of the PAHs and ratios lower than 0.4 would suggest petrogenic sources and higher than 0.4 combustion processes. As can be seen, only the samples collected in the port in Tromsø show this feature, and this could be linked to diesel combustion or road dust, among others (Tobiszewski and Namieśnik, 2012). Finally, though the ratio Phenanthrene/Anthracene is not included in Fig. 1, it also suggest a clear pyrogenic source, since the ratios are $\ll$ 10. A ratio close to the threshold limit of 10 is only observed for the farmed mussels.

PCA analysis of chemical burden in soft tissues of mussels showed that the sites defined as reference sites in the present work are different to the sites located in the ports. In addition, seemingly mussels from the ports were affected by different mixtures of metals and PAHs and exhibited different responses in certain biomarkers like in the case of LSC. Mussels from the WWTP presented low levels of contaminants, similar to the levels of mussels from the reference sites as seen in the PCA analysis. Thus, the different biological status of mussels sampled in the vicinity WWTP site is probably related to the presence of other kind of pollutants and/or other ecological factors, as discussed below.

Enzymatic activity can be affected by seasonal changes and environment, e.g. when the organism is actively feeding and thus is more exposed to the pollutant uptake (Nahrgang et al., 2013; Benito et al., 2019). Catalase activity did not differ between reference sites and ports. Thus, biomarker levels measured in the present work could be representing the basal state of the antioxidant activity which would be sufficient to cope with mild chemical stress. This can be in accordance to the guidelines by Molvær et al. (1997) which observed that chemical content in mussels was generally present at low to medium concentrations at which the effects are possible but not necessarily exhibited. In this case it is noteworthy that the enzymatic biomarkers were not affected by the geographical location of the mussels. Likely, environmental conditions, such as food availability, differ between the two locations and among other factors, nutritional status is known to affect antioxidant expression (González-Fernández et al., 2015a; 2015b). CAT levels reported by Storhaug et al. (2019) in mussels sampled in the lower intertidal zone from Tromsø in September–November were comparable to the present data, which might suggest that the current results are indicative of a non-impacted situation at least in mussels from Tromsø. With respect to the factors discussed above and the different condition of gamete development found in mussels between sites, it was interesting to find out that protein content in digestive glands of mussels did not differ between geographical regions. Conversely, significantly lower concentrations in chemically impacted sites in each region were obtained when compared to their relative reference site. Although no obvious correlations between the chemical burden and protein content were observed, it is plausible that lower protein concentrations are consequence of chronic exposure to mild chemical stress. Similar decrease in protein content in the digestive gland as an effect of pollutant exposure in mussels (*Mytilus galloprovincialis*) was demonstrated by Dobal et al. (2022), where the decrease was correlated with both increasing chemical concentration and time of exposure. If this was the case, then protein content could serve as a more sensitive response to chronic exposure in populations that have adapted to mild chemical concentrations in their environment compared to enzymatic biomarker responses. Interestingly, the protein content trend seemed to be opposite to the trend shown by CTD levels, as the groups with the lowest protein concentration in digestive gland presented higher CTD values. The loss of digestive gland integrity represented by higher CTD values is based on the lysis and resorption of digestive tissue (Brooks et al., 2011), which could be reflected in the decrease of the total protein content of the organ due to the proportionally minor amount of digestive and basophilic cells.

Mussels from the WWTP at Trondheim exhibited a dissimilar gonadal stage in comparison with the others from the same geographical area: they were mostly in advanced gametogenesis. It is expectable to find differences in the physiology of mussels that are near to a WWTP effluent due to increased food availability and/or the effect of pollutants that have been previously described as a result of WWTP discharges (Dumas et al., 2020; Preisner et al., 2021). Mussels from the reference site and the port in Trondheim could have performed a secondary spawning in late summer and at the time of this sampling could be facing the start of winter with immature gametes or it could be the start of a winter maturation (Duinker et al., 2008). The gonadal stages described in mussels from Tromsø, advanced gametogenesis or even mature gametes, could be indicative of an upcoming late autumn spawning process or a more advanced winter maturation process which seems to be a seasonal phenomenon previously reported in the area (Fokina et al., 2018; Storhaug et al., 2019). The factors influencing the differences in gamete developmental stages are to be taken into account as they could interfere heavily with the biomarker responsiveness (Beyer et al., 2017; Benito et al., 2019).

An overall higher amount of neutral lipids in digestive cells (Vv_{NL}) was found in mussels collected from reference sites in Tromsø than in Trondheim, which is coherent with previous studies carried out with mussels from arctic latitudes (Fokina et al., 2018). Mussels from the port in Trondheim and Tromsø presented lower Vv_{NL} values when compared to mussels from the reference sites in the same locality, which could be explained as a depletion of lipids under slight chemical stress (Guerlet et al., 2007) and more precisely by mild metal pollution (Zorita et al., 2006). Previous studies in the Bay of Biscay (Garmendia et al., 2010) reported reference Vv_{NL} values of 0.05–0.1 $\mu m^3/\mu m^3$ in October which is only comparable with the results obtained in Trondheim in the present work. Mussels from the port in Tromsø presented higher or similar Vv_{NL} values to the high values of the Bay of Biscay range, while lipid content in mussels from the reference site were well above the described range. These results are concordant with previous works (Brooks et al., 2015) and they confirm that Norwegian *M. edulis* naturally display higher Vv_{NL} values than *M. galloprovincialis* from the Bay of Biscay. The lower Vv_{NL} and ADG index levels described in mussels from the Norwegian ports may be indicative of mild chemical stress affecting mussels inhabiting these areas.

Lysosomal membrane stability test has been widely used as an indicator of general stress in mussels (Izagirre and Marigómez, 2009; Benito et al., 2019; Blanco-Rayón et al., 2019a). Accordingly, in the present study, LMS displayed the lowest values in mussels from the ports of Trondheim and Tromsø, while the mussels from the WWTP showed intermediate values between the ones present in the polluted and the reference sites. Although the measured responses are in concordance with the relative burden of pollutants in the tissues, both the highest and the lowest values are below what it was expectable taking into account the mild pollutant concentration in tissues. Threshold levels in mussels from the Bay of Biscay are established in >20 min for pristine environmental conditions (Marigómez et al., 2013), but in the present study the reference sites and the WWTP present values that are between 10 and 20 min which correspond to tolerable environmental condition. Mussels sampled in the ports are, on the other hand, in the range of delicate environmental condition (5–10 min). These results are in concordance with previous studies that compared the response of certain biomarkers in Norwegian *M. edulis* with Basque *M. galloprovincialis* (Brooks et al., 2015), where slightly lower LP were measured in Norwegian mussels compared to Basque mussels. However, it is not possible to apply the thresholds that are established for the Bay of Biscay in the present study, as this is the first effort to define the baseline values of certain biomarkers in the sampled latitudes.

Regarding lysosomal structural changes, the lowest Vv_{LYS} values were measured in the Trondheim port, which presented small lysosomes in high numbers, this picture being in concordance with the responses to metal exposure in *M. edulis* (Brooks et al. (2015) or to a low concentration of organic contaminants (Etxeberria et al., 1994). The lack of differences between both sites in mussels from Tromsø could rely in a mixture of mild pollutant levels in the port together with the confounding effect of the high prevalence of *Gymnophallus* cf. *Bursicola* trematodes in this locality (see below). Maximum values registered in the current study regarding Vv_{LYS} are way above the threshold values described in the Bay of Biscay for bad environmental conditions (Marigómez et al., 2013), furthermore, the highest values are displayed by mussels from the reference sites in Trondheim and Tromsø. The implications of these results are still unknown although, the seasonal or geographical effects (Benito et al., 2019) or even the effect of undetected pollutants cannot be discarded. Though, the latter factor is not so probable to be the cause of the discrepancy due to the chemical characteristics of the sampling sites. Nevertheless it is noteworthy that previous studies (Brooks et al., 2015) reported higher Vv_{LYS} values in *M. edulis* than in *M. galloprovincialis*, being comparable to the ones reported in the present study. LSC proved to be a valuable tool to assess general stress responses in wild mussels in the North Atlantic Sea, but in order to link alterations in this endpoint to the effect of pollutants it is necessary to clarify other confounding factors such as parasitism and different threshold levels found naturally under certain seasonal and latitudinal circumstances.

At higher complexity levels of biological organization, the three tissue level biomarkers used in the present work displayed similar trends. Vv_{BAS} was highest in mussels from the port in Trondheim which could be related to chemical stress (Blanco-Rayón et al., 2019b). Although Vv_{BAS} values in that group are remarkably high for the relatively mild pollutant levels in tissue when compared to the Bay of Biscay thresholds (Marigómez et al., 2013). Brooks et al. (2011) demonstrated that Norwegian mussels can reach high Vv_{BAS} levels without the need of a grave chemical insult. In addition, significant differences were detected between the individuals sampled in the farm and the ones from the WWTP, the latter presenting enhanced Vv_{BAS} values, which could be related to chemical insult and/or higher parasitic burden (discussed below). The lowest values in the present study were above 0.1 $\mu m^3/\mu m^3$ which is the good environmental condition threshold in the Biscay Bay (Marigómez et al., 2013). Thus, it could be concluded that the Vv_{BAS} baseline values and its responsiveness towards pollution for Norwegian mussels are higher than the ones established for mussels from the Bay of Biscay, this being in concordance with data reported in previous studies (Brooks et al., 2011).

Lowest values of CTD ratio were measured in mussels collected in the reference site in Trondheim. The response displayed by mussels from the Trondheim port could be caused by chemical insult (Múgica et al., 2015), in addition, the fact that the only statistically significant differences regarding ADG cell density were detected in mussels from the port in Trondheim could indicate the effect of a moderate chemical insult and a subsequent reduction of the storage material (Moukrim et al., 2008). The high CTD ratio measured in mussels from the WWTP could be caused by the effect of parasitism. The intermediate values registered in farmed mussels could be caused by a decreasing dietary condition (Benito et al., 2019), since mussels were collected from the depuration tank, where food is not available. The highest atrophy index values can be considered as mild levels of digestive tissue degradation, which in the case of mussels from the port in Trondheim were significant when compared to the ones from the reference site in the same locality. This is concordant with data of pollutant burden in tissues. Mussels from the reference site in Tromsø displayed significantly high atrophy levels when compared to mussels from the reference site in Trondheim. This could be related to geographical and seasonal effects (Benito et al., 2019) and/or parasitic burden (Cuevas et al., 2015). Mussels from the port in Tromsø displayed similar atrophy levels to the ones from the reference site, the cause could be an insufficient concentration of pollutants in the site to exert a severe response in this biomarker combined with the masking effect that parasitism could be causing in both groups. It can be concluded that the present atrophy index and CTD ratio data is valuable as a first step towards the establishment of the thresholds indicating good health status and/or general stress conditions in mussels from the studied areas.

Among the remarkable parasitic content, mussels from the WWTP presented relatively high prevalence of *Renicola* sp. Trematodes. It has been demonstrated that mussels collected from benthic intertidal beds present higher parasitic burden than the ones collected from ropes, pylons and similar vertical structures (Benito et al., 2022). In Trondheim, the only mussels collected from the benthic intertidal habitat were the ones from the WWTP, while the ones from the reference site and the port were collected from pylons, and the ones from the farm were collected from long lines. In addition, the potentially increased organic matter in the WWTP effluent (Preisner et al., 2021) and the subsequent increased amount of prey (Wołowicz et al., 2006) (at least in the vicinity of Trondheim WWTP) attract bigger numbers of mussel-eating seabirds (personal communication by Tomasz M. Ciesielski) that are the final host of *Renicola* sp. Trematodes (Stier et al., 2015). Infection of trematodes from the Renicolidae family are known for causing impairments in mussels regarding clearance rate and even growth rate, but mostly when environmental conditions for the hosts are not optimum as they act as background stressors (Thieltges, 2006; Stier et al., 2015). A sampling site that presents an important trematode prevalence like the WWTP in Trondheim must be approached with caution when assessing the stress levels of mussels via biomarkers, as certain responses could be at least partially caused by parasitic burden and not by chemical stressors. Mussels from Tromsø presented trematode infection too, although *Renicola* sp. Parasites were only found in the reference site. However, both sites presented *Gymnophallus* cf. *Bursicola* parasites that elicited a significant immune response from the host (Benito et al., 2022). Furthermore, mussels infected with the *Gymnophallus* cf. *Bursicola* trematode presented different combinations of additional lesions in the mantle that ranged from haemocytic infiltrations in the connective tissue and/or gonad follicles, atresia and brown cell infiltrations in male follicles (lesser extent). Although the host response is similar in both bivalves, the infection intensity is higher in cockles (Goater, 1993) than what is found in mussels in the present study. As it has been already discussed, *Renicola* sp. Are known to be a background stress source, even with an almost nonexistent histopathological host-response, thus, the pathogenicity of the *Gymnophallus* cf. *Bursicola* parasites in the present study seems to be of critical importance having into account the host-response it generates. The stress caused by the immunological response (tissue encapsulation, inflammatory responses) could explain the stress levels found in certain biomarker responses in mussels from the allegedly non-impacted site in Tromsø including lysosomal alterations and high atrophy and CTD levels.

Regarding pathological status in mussels sampled in Trondheim the most remarkable results are a higher prevalence of haemocytic infiltrations in mussels form the port and from the WWTP. It is known that these inflammatory responses are related to starvation, reproductive stress, shell damage, parasitism and exposure to hydrocarbons and metals (Garmendia et al., 2011; Benito et al., 2019). Exposure to chemical stressors is the most probable cause of the haemocytic infiltrations found in mussels from the Trondheim port, while mussels from the WWTP might show a high prevalence of inflammatory responses as an outcome of the combination of mild chemical insult and parasitism. In the case of mussels from Tromsø the increased prevalence of haemocytic infiltration could be related to the presence of trematodes as discussed before. The presence of brown cells in gonad follicles and atresia of oocytes could be indicators of ongoing autolysis and resorption processes during gametogenesis, and can be induced when environmental conditions become unfavorable for spawning after gamete maturation (Suárez et al., 2005; Smolarz et al., 2017). Thus, it is expectable to find these alterations more commonly in individuals with a more advanced gametogenic state like mussels from the WWTP in Trondheim and mussels sampled in Tromsø. Although the presence of atresia is not necessarily pathological, it seems that the presence of the *Gymnophallus* cf. *Bursicola* trematodes might be one of the causes behind the increase of atresia prevalence in mussels from Tromsø. Granulocytomas are inflammatory responses resulting in vascular occlusions (Lowe and Moore, 1979) that have been linked to chronic pollution and the presence of metacercarian parasites (Garmendia et al., 2011; Benito et al., 2019). The latter seems to be the differential reason to explain the relatively high granulocytoma prevalence in Tromsø as the higher pollutant concentration found in the port did not seem to increase the appearance of these lesions. This reinforces the idea that the *Gymnophallus* cf. *Bursicola* parasite is a variable to take into account as a confounding factor to assess the environmental health status by biomarker responses and histopathology which is coherent with previous studies (Benito et al., 2022).

Even though the interpretation of the responses of single biomarkers does not indicate a clear chemical insult under the current environmental conditions, it is known that integrative tools like the IBR index are helpful to accentuate diffuse stress signals in a holistic way, as it has been reported before (Marigómez et al., 2013). In the present study, although the effect of environmental pollution was clearly reflected in relative terms by the IBR index (as the mussels from the ports displayed higher stress responses and IBR/n), the effects of the confounding factors described before were also appreciated in mussels from the WWTP and the reference site in Tromsø. Thus, it can be concluded that the application of the IBR index confirms the selected battery of biomarkers as an efficient tool in order to discriminate mussel populations inhabiting locations chronically impacted by mild pollution from mussels collected in chemically non-impacted sites despite all the confounding factors generated by environmental conditions.

5. Conclusions

The present study is the first step towards the establishment of baseline level values of biochemical, cell and tissue-level biomarkers and their responsiveness to pollutants in two areas at subarctic and Arctic latitudes. Although the battery of biomarkers used here proved to be useful to discriminate chemically impacted and non-impacted mussel populations, some confounding factors altering the biological responses were identified. Hence, geographical/latitudinal factors seem to be critical regarding the reproductive cycle, reserve material storage and the prevalence of parasites. As in the case of mussels from the reference site in Tromsø that displayed general stress responses at different levels, which could have been influenced by the pathogenic effect of the *Gymnophallus* cf. *Bursicola* trematode and/or by a more advanced gametogenic developmental stage, which could lead to the misinterpretation of the assessment of the effect of environmental pollutants in those mussels. Overall, the current work serves as an anchor point both as a reference of the baseline level values of the analyzed endpoints in the studied geographical area and time of the year, and as an indication of the potential extent of the environmental confounding factors causing stress on the analyzed mussel populations.

Author statement

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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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Appendix A. Supplementary data

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