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Variability and distribution of parasites, pathologies and their effect on wild mussels (*Mytilus* sp) in different environments along a wide latitudinal span in the Northern Atlantic and Arctic Oceans

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ABSTRACT

Histopathological examination in mussels can provide useful information for the diagnosis of ecosystem health status. The distribution of parasites in mussels can be conditioned by several environmental factors, including mussels collecting sites or the presence/absence of other species necessary to complete the complex life cycle of certain parasites. Thus, these variables could not only govern the parasitic burden of mussels but also the presence of pathologies associated to parasitism. The aim of this study was to identify the histopathological alterations which could be indicative of a health status distress along a wide latitudinal span in the Northern Atlantic and Arctic Oceans in mussels of two size-classes sampled in clean and impacted sites. A latitudinal gradient is clearly observed in gamete developmental stages as northern and southern mussels presented different conditions at the same period. Furthermore, mussels of the same size in different latitudes presented differences in the reproductive cycle and the appearance of related pathologies, which probably meant the age of individuals was different. In addition, specific parasitic profiles ruled by latitudinal conditions and the settlement of mussels in the shore (horizontal/vertical) have been demonstrated to be significantly influential in the health condition of mussels. Furthermore, the present work provides the first histological description of *Gymnophallus* cf. *bursicola* parasite causing a considerable host response in Tromsø and Iceland plus the report of grave histopathological status that included high prevalence of granulocytomas in Scotland and Germany.

1. Introduction

Mussels (*Mytilus* sp.) are widely used sentinel organisms in pollution monitoring programs to determine the health status of coastal ecosystems and the use of the biomarker approach has been widely established (Brenner et al., 2014; Nasci et al., 2002). Even though this approach has been proved to be useful, the interpretation of biological responses related to chemical insult can be biased due to the interactions between pollutants and natural factors or between natural and physiological factors. Consequently, these possible confounding factors must be precisely identified (Benito et al., 2019; Beyer et al., 2017; Fokina et al., 2018; Leiniö and Lehtonen, 2005; Nahrgang et al., 2013). In addition, the definition of the range of natural variability and how it may influence on the correct interpretation of the biological effects caused by pollution is of mayor importance (Beyer et al., 2017; Izagirre et al., 2008).

Histopathological examination in mussels can provide useful information for the diagnosis of ecosystem health status (Garmendia et al., 2011). Changes in the parasitic burden and the development of inflammatory and degenerative lesions, which are among the most common histopathological abnormalities, can be caused by disturbances at low levels of biological complexity and may pose deleterious consequences for the health status of populations (Moore and Simpson, 1992). Even though histopathology in mussels has been previously used for the assessment of the effects of exposure to PAH, PCB and metals (Auffret, 1988; Lowe and Pipe, 1987; Marigómez et al., 2006), care must be taken when directly using histopathology as a biomarker of exposure since it demonstrated to be dependent on parasite infestation of wild mussel

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populations (Bignell et al., 2008, 2011; Cuevas et al., 2015; Villalba et al., 1997).

The distribution of parasites in mussels can be conditioned by several environmental factors, including mussels collecting sites (Buck et al., 2005) or the presence/absence of other species necessary to complete the complex life cycle of certain parasites (Goater, 1993). Thus, these variables could not only govern the parasitic burden of mussels but also the presence of pathologies associated to parasitism. In addition, the reproductive cycle of mussels is another important confounding factor among that is known to influence biomarker responsiveness (Benito et al., 2019), which at the same time it is known to be influenced by seasonality (Sheehan and Power, 1999) and age of mussels (Handå et al., 2011).

The aim of this study is to identify the histopathological alterations that could be indicative of a health status distress that might potentially cause an altered biological response influencing the biomarker responsiveness to pollutants on mussels from the Northern Atlantic Ocean and Arctic Ocean, along a wide latitudinal span. For this purpose, a histopathological assessment was carried out analyzing the gamete developmental stages, the presence of parasites and the appearance of pathologies in the mantle and digestive gland of mussels. Furthermore, the possible relationships between the presence of parasites and the appearance of pathologies were established and the most influential environmental factors on the prevalence of the histopathological abnormalities were identified. The potential influencing factors taken into account in the present study were anthropogenic impact, reproductive cycle, mussel size as a proxy of age, collection site characteristics and geographical issues. The possible pathogenicity of the histopathological alterations and its potential effect on biomarker responsiveness are also discussed considering the implications these variables might have on classic biomonitoring design guidelines.

2. Material and methods

2.1. Sampling strategy

Mussel collection was carried out in late summer 2017 at different locations of the northern Atlantic Ocean and Arctic Ocean along a wide latitudinal span (Fig. 1).

The sampling sites where mussels were collected are described in Table 1, including GPS coordinates, date of collection, type of habitat, mussel settlement in the spot (vertical versus horizontal) and their classification according to latitudinal categories. Latitudinal categories were defined as Northernmost locations (sampling sites above the arctic circle), Intermediate locations (sampling sites located between the arctic circle and the 60° N parallel) and Southernmost locations (sampling sites located below the 60° N parallel).

In order to compare sampling sites depending on their anthropogenic impact (non-impacted versus impacted), sites were selected following the advice of local researchers that use mussels from these sites in biomonitoring programs or with research purposes. Thus, the non-impacted sites (in green, Table 1) are relatively low populated open (high water renewal rate) natural environments. On the other hand, impacted sites (in yellow in Table 1) are more heterogeneous: Eckwarderhörne is located in the mouth of an enclosed bay located in the vicinity of Wilhelmshaven and Bremerhaven, which are relatively highly populated cities with important shipbuilding and commercial port activities. Similarly, Leith is located in the harbour area of Edinburgh and it may also be impacted by other human activities such as agriculture, oil industry and a nuclear plant. The sampling site of Malmøya is an enclosed marina in Oslo while wastewater treatment plant (WWTP) in Trondheim, corresponded to a rocky beach close to a wastewater treatment plant effluent. The rest of the impacted sampling sites in Trondheim, Tromsø and Reykjavik were located in enclosed ports.

Mussels of two different sizes (small: 2–3 cm and large: 3.5–4.5 cm) were sampled, transported to the laboratory in air at ambient temperature and dissected immediately except in the two German sampling

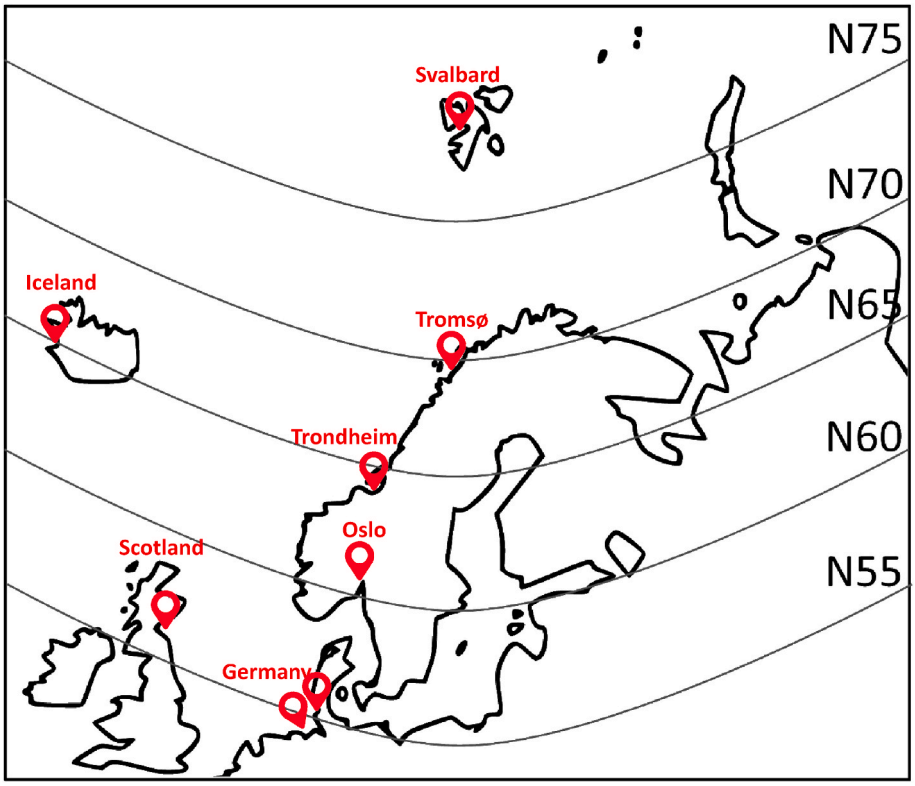

Fig. 1. Map indicating the geographical location of the sampling sites.

Table 1
Precise information of the sampling sites including anthropogenic impact, geographical location, date of sampling, tidal regime, characteristics of sampling sites, distribution of mussels and the latitudinal clusters in which they were classified. Green: non-impacted, yellow: presence of important anthropogenic impact. H: Horizontal, V: Vertical, S: Southernmost, I: Intermediate, N: Northernmost.

Country	Location	Latitude	Longitude	Date	Tidal regime	Sampling site type	Settlement of mussels	Latitudinal cluster
Germany	Königshafen	55.042374	8.449102	October 10, 2017	Intertidal	Mussel bed	H	S
	Eckwarderhörne	53.520301	8.231816	October 11, 2017	Intertidal	Rock breakwater	H	S
Scotland	St. Andrews	56.333602	−2.776173	September 21, 2017	Intertidal	Rocky beach	H	S
	Leith	55.977359	−3.140404	September 20, 2017	Intertidal	Rocky beach	H	S
Iceland	Havfjördur	64.358154	−21.486458	September 07, 2017	Intertidal	Rocky beach	H	I
	Reykjavik (port)	64.155605	−21.939218	September 08, 2017	Intertidal	Rock wall	V	I
Norway	Oslo (Malmøya)	59.873896	10.757743	September 15, 2017	Subtidal	Floating jetty	V	S
	Oslo (Drøbak)	59.615889	10.652007	September 18, 2017	Intertidal	Rocky beach	H	S
	Trondheim (port)	63.442692	10.425494	September 20, 2017	Intertidal	Pylons	V	I
	Trondheim (WWTP)	63.444867	10.341331	September 21, 2017	Intertidal	Rocky beach	H	I
	Trondheim (Rissa)	63.561753	9.899776	September 21, 2017	Intertidal	Pylons	V	I
	Tromsø (port)	69.654177	18.968459	September 18, 2017	Intertidal	Concrete surface	H	N
	Tromsø (aquarium)	69.642089	18.94639	September 18, 2017	Intertidal	Rocky beach	H	N
	Svalbard (Longyearbyen)	78.236081	15.606482	September 21, 2017	Subtidal	Pylons	V	N

point where they were dissected *in situ*. All the collected mussels were intertidal, picked up from the first meter of the lower intertidal zone except in Malmøya (Oslo) and Svalbard where the mussels were subtidal and retrieved from a floating jetty and underwater by scuba diving, respectively.

2.2. Sample processing

Transversal slices of 20 mussels in each sampling site and size including digestive gland, gills and mantle were made for histopathological analysis. They were fixed in seawater with 4% formaldehyde, dehydrated in an ethanol bath series, paraffin embedded using a Leica ASP3005 tissue processor, sectioned serially at 5 μm with a Leica RM2125RTS microtome and stained with hematoxylin-eosin for histopathological analysis.

2.3. Gamete developmental stages

Gamete developmental stages were determined in histological preparations (n = 20) as described by Ortiz-Zarragoitia et al. (2011) and were distinguished in mussel gonads as follows: resting stage (inactive or undifferentiated); early gametogenic stage (gametogenesis has begun but no ripe gametes visible); advanced gametogenic stage (gametogenesis still progressing and ripe gametes and developing gametes have about equal proportions); mature stage (gonad fully mature, follicles full of ova or sperm); spawning stage (active emission of gametes, some follicles appear empty); post-spawning stage (empty follicles and only residual gametes remain).

2.4. Histopathological analysis

Slides of 20 mussels per sampling campaign were examined individually under the light microscope using 10 ×, 20 × or 40 × objective lenses. Quantitative scores were made by keeping a running count of the occurrence as the slide was scanned to avoid re-examination of each slide multiple times for each category. The prevalence of intracellular microcolonies of bacteria (IMB), intracellular ciliates (IC) in the epithelium of digestive tubules, *Mytilicola* sp. copepods, trematode sporocysts (Bucephalidae) and trematode metacercariae (presumably, *Gymnophallus* cf. *bursicola* and *Renicola* spp. trematodes) was analyzed as indicated by Kim et al. (2006). In addition, the prevalence of certain tissue conditions was analyzed, including cases of brown cell (ceroid bodies or pigment cells) aggregates in digestive gland and gonad, follicular atresia, granulocytomas and haemocytic infiltration in digestive gland and gonad, without distinction between focal and diffuse. The intensities of IMB, IC, *Gymnophallus* cf. *bursicola* and *Renicola* spp. trematodes and the severity of granulocytomas were calculated quantitatively. The obtained scores were used to compute the following parameters: Prevalence = N_H/N_S, and Mean Intensity = S_P/N_H; where N_H is the number of specimens hosting parasites or pathologies, N_S is the number of specimens analyzed per sample, S_P is the score corresponding to each parasite and pathology recorded. Prevalence and intensity provide information about the occurrence of each parasite and tissue condition.

The intensities of trematode sporocyst (Bucephalidae) infection and atresia were assessed as described by Kim et al. (2006).

Trematode sporocyst infection intensity:

0 Uninfected
1 Present in the gonads only (some gametic tissue still present)
2 Completely filling the gonads (no gametic tissue present); may be present in digestive gland or gills in very limited amount
3 Completely filling the gonads; extensive invasion of the digestive gland and/or the gills
4 Completely filling the gonad; substantially filling the digestive gland or gill; individuals appear to be a sack of sporocyst.

Atresia severity:

0 Normal gonad
1 Less than half the follicles are affected
2 About half the follicles are affected
3 More than half the follicles are affected
4 All follicles affected

2.5. Statistics

Statistical analysis was carried out with the aid of the SPSS/PC + statistical package V.24 (SPSS Inc., Microsoft Co.). After data analysis, Pearson's test ($P < 0.01$, $P < 0.05$) was selected for the correlation of the prevalence of different parasites and pathologies. Kruskal-Wallis test was used to compare the distribution ($P < 0.05$) of the prevalence of parasites and pathologies when sampling groups were clustered depending on sampling sites with or without high anthropogenic activity, characteristics of the substrate in which mussels were sampled, size of the mussels and latitudinal categories.

3. Results

3.1. Gamete developmental stages

In general terms, mussels from all sites showed signs of an ongoing spawning process or a recent spawning event (Fig. 2). However, it should be noted that in southern sites higher percentage of post-

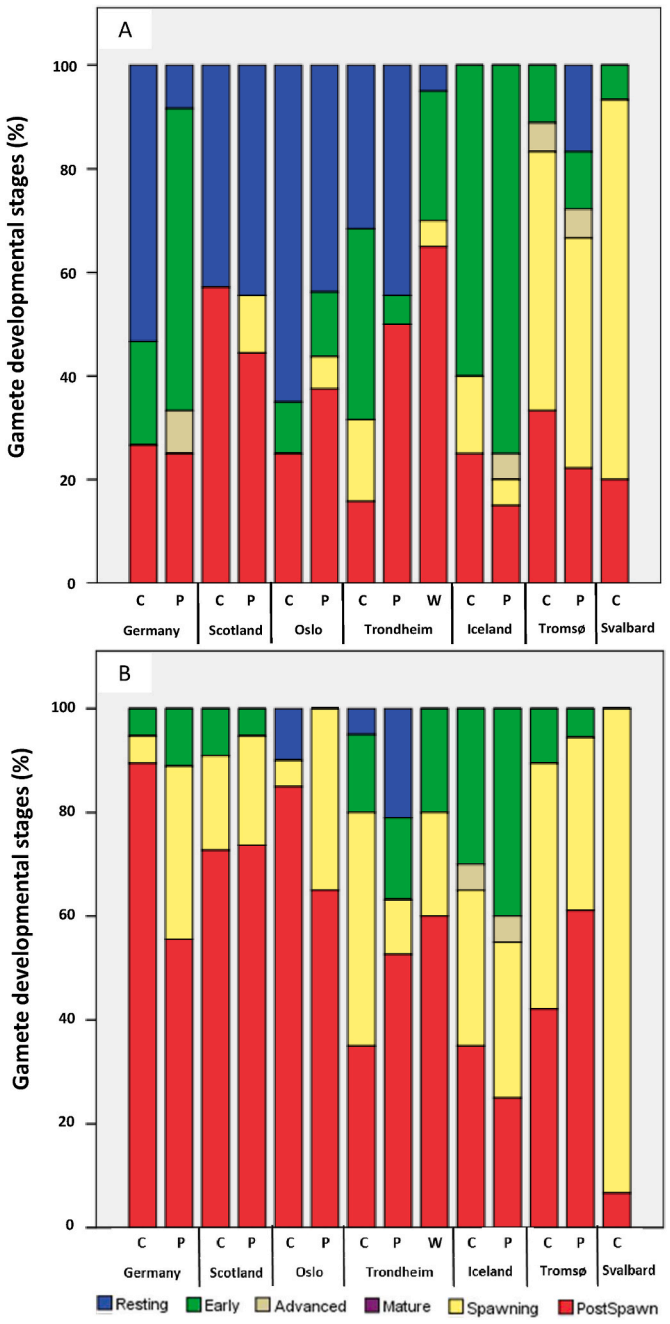

Fig. 2. Graph represents percentages of gamete developmental stages in small mussels (A) and in large mussels (B). Letters indicate anthropogenic impact: C: Clean, P: Presence of Anthropogenic activities.

spawned mussels was observed while in the northern sites percentage of spawning (Fig. 3A and B) mussels was higher. Comparison between size (age) groups showed certain differences can also be appreciated when comparing mussel sizes, as the sum of the percentages of mussels spawning and post-spawning in small mussels was around 50% or below with the exception of mussels from the WWTP in Trondheim, Tromsø and Svalbard. Opposite, large mussels presented a sum of percentages of spawning and post-spawning stages close to 80% or above, with the exception of Icelandic mussels exhibiting values around 50–60%.

3.2. Parasite and histopathological alterations: prevalence and mean intensity

Regarding parasite infection the prevalence of microorganisms (Table 2) such IMB in the epithelium of the tubules of the digestive gland of mussels (Fig. 4A) was overall low, being small mussels from the clean site in Oslo the group with the highest prevalence (20%). Overall, IC (Fig. 4B) prevalence was below 20% except in large mussels from the harbour in Trondheim where more than 50% of the individuals exhibited these intracellular ciliates. The prevalence of *Mytilicola* sp. (Fig. 4D) was high in mussels sampled in Germany (33%–66.7%), but the prevalence was low (<15%) in the rest of the groups that presented this copepod. On the contrary, trematode sporocyst infection (Bucephalidae) (Fig. 4C) was only found in 3 groups and the prevalence was also low (<11%). *Gymnophallus* cf. *bursicola* parasites (Fig. 4E and G) were only found in Tromsø and Iceland, and except in large mussels from the clean site in Tromsø, where the prevalence was 31.58%, the rest of the groups presented lower prevalence (<16%). *Gymnophallus* cf. *bursicola* metacercariae (ca. 250–500 μm in diameter) was found in the mantle surrounded by columnar epithelium cells from the pallial line. In the case of the trematodes that were totally enclosed by the host epithelium, haemocytic activity (that would indicate the start of the lysis of the parasite) was also present, which is coherent with the cellular debris also observed inside the host-tissue capsule. In addition, mechanical disruption of gonad follicles seemed to be common (Fig. 4E and G). The prevalence of *Renicola* spp. trematodes (Fig. 4F) in those groups where it was found ranged between 35 and 76.47% except in the clean site of Tromsø, where it reached only to the 5.26%, these parasites were principally found in the digestive gland, but in the case of mussels from Germany, they were also found in the foot occasionally.

IMB mean infection intensity was overall low, the impacted site large mussels from Oslo being the exception with up to 134 microcolonies present per histological section. All the IC ciliates counts were below 50 specimens per histological section. Trematode sporocyst (Bucephalidae) mean infection intensity was high in large mussels from the clean site in Scotland and small mussels from the clean site in Tromsø, and intermediate in small mussels from the impacted site in Tromsø. Only one metacercariae of *Gymnophallus* cf. *bursicola* trematode was found in most of the infected mussels except in the large mussels sampled in the clean site in Iceland and in the impacted site in Tromsø which mean infection intensity was of 2 metacercariae per histological section. *Renicola* spp. trematode mean infection intensity ranged from 1 to 5 metacercariae per histological section.

Regarding other pathologies, brown cell infiltration in gonad (Fig. 3D) was found in a relatively small amount of individuals. The highest prevalence (33.33%) was detected in large mussels from the impacted site in Germany while for the rest of the groups it ranged from 0 to 20%. Atresia (Fig. 3A) was prevalent in both small and large mussels from Svalbard 88.38% and 100% respectively, followed by mussels from the clean site in Tromsø (50% in small mussels and 54.55% in large mussels) Atresia levels ranged from 0 to 42% for the rest of the groups. Highest prevalences of haemocytic infiltration in mantle tissues were seen in large mussels from the impacted site in Oslo and in the clean site in Iceland (both 50%), followed by the impacted site in Scotland and the clean sites in Tromsø (47.37%), the clean site in Oslo (35%) and finally Scotland (28.57%). The rest of the groups displayed a prevalence ranging from 0 to 20%. The prevalence of brown cell infiltration in the digestive gland (including digestive alveoli and digestive tract epitheliums) (Fig. 3C) was low (<11%) in small mussels from Scotland, small mussels from the WWTP in Trondheim, small mussels from the clean site in Tromsø and large mussels from the clean site in Iceland, while in the rest of the groups it was much higher with values ranging from 15% to 70%. Granulocytomas consist of a mass of accumulated granulocytes surrounded by layers of flattened, epithelioid cells (Fig. 3E), and the highest prevalences were found in large mussels from the impacted site in Germany (50%) and large mussels from the impacted and clean sites in Scotland (47.37%, 42.86% respectively). The rest of the groups in which granulocytomas were detected displayed a prevalence of less than 17%. The highest haemocytic infiltration prevalence in digestive gland and gills was found in small mussels from the impacted site in Oslo, large

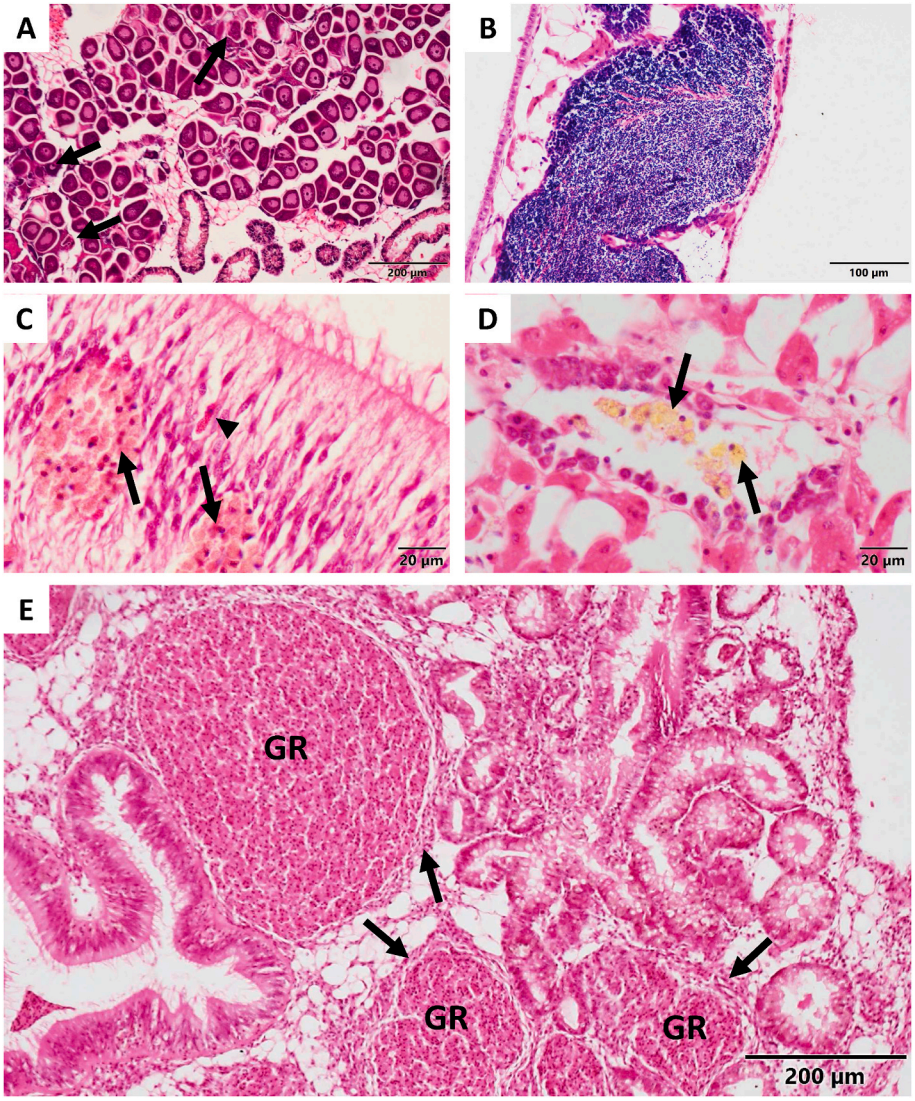

Fig. 3. Micrographs (Hematoxylin-Eosin staining) showing A: Spawning process of a female gonad and atresic oocytes (arrows) in a mussel from Svalbard. B: Spawning process of a male gonad in a mussel from Svalbard. C: Brown cell infiltration (arrows) and a haemocyte (arrowhead) in diapedesis in the digestive epithelium in a mussel from Germany. D: Brown Cell infiltration in gonad follicles (arrows) of a mussel from Germany. E: Multiple granulocytomas consisting of a mass of accumulated granulocytes (GR) surrounded by layers of flattened, epithelioid cells (arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mussels from Germany, the four groups from Scotland, both groups from the WWTP in Trondheim, small mussels from the clean site in Tromsø and small mussels from the impacted site in Iceland, all of them ranging from 64.29% to 78.95%. Mussels from the clean site in Oslo, large mussels from the impacted site in Oslo, small mussels from Germany, mussels from the clean site in Trondheim, all groups from Tromsø except small mussels from the clean site and both groups of large mussels from Iceland displayed prevalence ranging between 20% and 55.56%. The rest of the groups displayed a haemocytic infiltration prevalence ranged from 0 to 16.67%. Mean atresia intensity was high in large mussels from the impacted site in Oslo (4), the impacted site in Germany (3), the clean site in Trondheim (3), large mussels from Svalbard (3.5) and small mussels from Svalbard (3.38). Mean atresia intensity was intermediate in large mussels from the WWTP in Trondheim (2), small (2.62) and large (2.42) mussels from the clean site in Tromsø, large mussels from the impacted site in Tromsø (2.17), large mussels from the clean (2.5) and impacted (2.83) sites in Iceland. The rest of the groups that displayed atresia presented low intensity (1). Large mussels from the clean site in Scotland presented more than 10 granulocytomas per slide in several individuals. Large mussels from the clean and impacted sites in Germany and from the impacted site in Scotland presented multiple granulocytomas (1–10) per slide, while mussels from the rest of the groups presenting this pathology displayed a single granulocytoma per slide.

3.3. Correlation analysis between the prevalence of parasites and histopathological alterations

Pearson's correlation (Table 3) showed significant correlations between the prevalence of *Mytilicola* sp. and *Renicola* spp. trematodes. The prevalence of *Gymnophallus* cf. *bursicola* trematodes was positively correlated with haemocytic infiltration in gonad while the prevalence of *Renicola* spp. trematodes was also positively correlated with the granulocytoma prevalence in the digestive gland and mantle. The prevalence of brown cell infiltration in the digestive gland was correlated with the prevalence of brown cell infiltrations in gonad and with granulocytoma prevalence while its prevalence was correlated with the prevalence of granulocytoma and hemocytic infiltration. The prevalence of granulocytomas was positively correlated with haemocytic infiltration prevalence in digestive gland.

3.4. Factors affecting parasites and histopathological alterations prevalence

Clean vs. impacted sites: No significant differences were found between mussels sampled in clean and impacted sites when comparing the prevalence of all parasites and pathologies (results not shown).

Vertical vs. horizontal settlements: Significant differences were found between mussels sampled from horizontal and vertical substrates

Table 2
Prevalence (%) of Intracellular Microcolonies of Bacteria (IMB), intracellular ciliates (IC), Mytilicola sp., Bucephalidae trematode sporocysts, Gymnophallus cf. bursicola and Renicola spp. trematodes, brown cell infiltration in gonad, atresia of the oocytes, haemocytic infiltration in mantle tissues, brown cell infiltration in digestive gland, granulocytoma and haemocytic infiltration in digestive gland. DG: Digestive gland, G: Gonad, M: Mantle.

Location	Impact	Size	IMB	IC	*Mytilicola* sp.	Bucephalidae	*Gymnophallus*	*Renicola* spp.	Brown Cell Inf.(G)	Atresia	Haem. Inf. (M)	Brown Cell Inf. (DG)	Granulocytoma	Haem. Inf. (DG)
Germany	Clean	Small	0	0	52.94	0	0	76.47	5.88	0	0	0	11.76	23.53
		Large	0	10	60	0	0	65	20	0	15	70	50	65
	Impacted	Small	0	0	66.67	0	0	16.67	0	0	0	0	0	16.67
		Large	0	0	33.33	0	0	38.89	33.33	9.09	5.56	61.11	16.67	66.67
Scotland	Clean	Small	0	21.43	0	0	0	0	0	0	14,29	0	0	64.29
		Large	0	14.29	0	7.14	0	21.43	7.14	0	28.57	35.71	42.86	71.43
	Impacted	Small	0	11.11	5.56	0	0	66.67	0	0	11.11	5.56	0	66.67
		Large	5.26	0	5.26	0	0	42.11	5.26	0	47.37	15.79	47.37	78.95
Oslo	Clean	Small	20	10	0	0	0	0	0	0	0	25	0	30
		Large	0	0	0	0	0	0	10	5.56	35	60	0	20
	Impacted	Small	0	0	0	0	0	0	0	0	20	25	0	65
		Large	5	5	0	0	0	0	5	7.14	50	50	10	45
Trondheim	Clean	Small	0	0	0	0	0	0	0	0	0	0	0	25
		Large	0	15	0	0	0	0	0	41.67	0	0	0	20
	Impacted	Small	0	15.79	0	0	0	0	0	0	0	0	0	0
		Large	0	52.63	0	0	0	0	0	0	0	0	0	5.26
	WWTP	Small	0	0	5	0	0	35	10	14.29	5	5	0	65
		Large	0	15	10	0	0	35	10	8.33	5	0	0	65
Iceland	Clean	Small	5	0	15	0	5	70	0	0	20	0	0	15
		Large	5	0	0	0	10	50	5	11.11	50	10.53	0	30
	Impacted	Small	0	0	0	0	0	0	5	0	5	0	0	65
		Large	0	0	0	0	5	0	0	25	5	0	0	25
Tromsø	Clean	Small	5.26	0	5.26	10.53	15.79	5.26	0	50	5.26	5,26	5.26	78.95
		Large	0	0	5.26	0	31.58	5.26	0	54.55	47.37	0	5.26	52.63
	Impacted	Small	5.56	0	0	5.56	0	0	0	11.11	0	0	0	22.22
		Large	5.56	0	0	0	5.56	0	16.67	30	5.56	0	0	55.56
Svalbard	Clean	Small	0	0	0	0	0	0	0	88.38	13.33	0	0	6.67
		Large	0	0	0	0	0	0	6.67	100	6.67	0	0	13.33

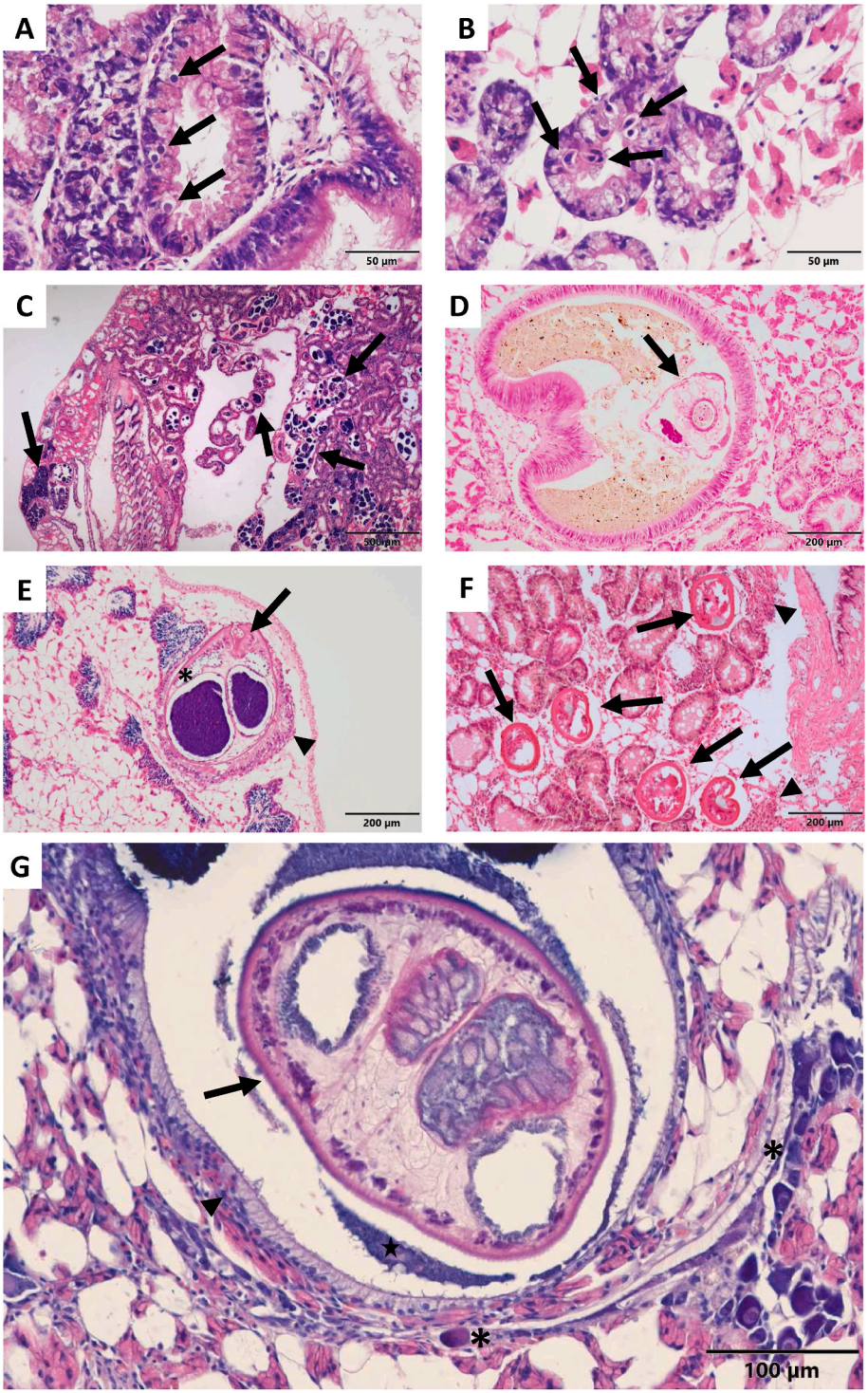

Fig. 4. Micrographs (Hematoxylin-Eosin staining) showing A: Intracelular Microcolonies of Bacterias (arrows) in digestive cells of mussel from Oslo. B: Intracellular Ciliates (arrows) in digestive cells of digestive alveoli of a mussel from Oslo. C: Bucephalidae trematode sporocysts (arrows) infecting mantle, kidney and digestive gland tissue in a mussel from Scotland. D: Mytilicola sp. (arrow) in the digestive tract of a mussel from Germany. Gymnophallus cf. bursicola trematode metacercariae (arrow), columnar epithelium of host mantle tissue (arrowhead) and mechanical disruption of the gonad follicle (asterisk) of a mussel from Iceland. F: Multiple Renicola spp. trematode metacercariae (arrows) infecting digestive gland tissue and immune responses of the host as focal haemocytic infiltration (arrowheads) in a mussel from Germany. G: Detailed view of a Gymnophallus cf. bursicola trematode metacercariae (arrow), columnar epithelium of host mantle tissue (arrowhead), cellular debris (star) and mechanical disruption of the gonad follicle (asterisks) of a mussel from Tromsø.

when comparing the prevalence of parasites and pathologies. The prevalences of *Mytilicola* sp. (Fig. 5A), *Renicola* spp. trematode (Fig. 5B) and haemocytic infiltration (Fig. 5C) in the digestive gland and the gills were higher in horizontal substrates than in vertical ones.

Size (age) effect: Larger mussels showed higher prevalence of infiltration of brown cells in gonad (Fig. 6A) and haemocytic infiltration of mantle tissues (B) than smaller mussels, while significant differences between size groups were not detected for the other parasites and pathological conditions.

Latitudinal effects: Mussels from northernmost locations exhibited significantly higher prevalence of *Gymnophallus* cf. *bursicola* trematodes (Fig. 7A) and atresia (Fig. 7C) while their counterparts from the southernmost locations exhibited significantly higher prevalence of brown cell infiltrations (Fig. 7B) and granulocytomas (Fig. 7D) in the digestive gland. Mussels sampled in intermediate latitudes showed intermediate values found in-between the ones observed in north and southernmost mussels for most of the prevalences and histopathological alterations.

4. Discussion

Histopathology in mussels has been widely used as an appropriate tool for assessment the environmental health status because

Table 3
Pearson's correlation for the prevalence of all the pathologies and parasites found. ** in green: Correlation is significant at the 0.01 level (2-tailed). * In yellow: Correlation is significant at the 0.05 level (2-tailed). IMB: Intracellular Microcolonies of Bacteria, IC: Intracellular Ciliates DG: Digestive gland, G: Gonad, M: Mantle.

		Bucephalidae	IC	*Mytilicola* sp.	*Gymnophallus*	*Renicola* spp.	Brown Cell Inf. (DG)	Brown Cell Inf. (G)	Atresia	Granulocytoma	Haem. Inf. (M)	Haem. Inf. (DG)
IMB	Correlation	0.137	−0.093	−0.186	0.054	−0.073	0.067	−0.128	−0.113	−0.033	0.057	−0.010
	Sig. (2-tailed)	0.489	0.638	0.344	0.785	0.710	0.733	0.516	0.568	0.867	0.775	0.960
	N	28	28	28	28	28	28	28	28	28	28	28
Bucephalidae	Pearson Correlation		−0.051	−0.125	0.228	−0.128	0.003	−0.123	0.114	0.203	−0.059	0.291
	Sig. (2-tailed)		0.798	0.527	0.243	0.516	0.988	0.534	0.565	0.301	0.766	0.134
	N		28	28	28	28	28	28	28	28	28	28
IC	Pearson Correlation			−0.136	−0.215	−0.124	−0.071	−0.158	−0.240	0.003	−0.211	−0.152
	Sig. (2-tailed)			0.490	0.273	0.531	0.720	0.422	0.218	0.989	0.281	0.441
	N			28	28	28	28	28	28	28	28	28
Mytilicola sp.	Pearson Correlation				−0.104	.561**	0.259	0.369	−0.233	0.353	−0.192	0.017
	Sig. (2-tailed)				0.599	0.002	0.184	0.054	0.232	0.065	0.328	0.930
	N				28	28	28	28	28	28	28	28
Gymnophallus	Pearson Correlation					−0.058	−0.194	−0.157	0349	−0.090	.390*	0.151
	Sig. (2-tailed)					0.771	0.323	0.426	0.069	0.648	0.040	0.442
	N					28	28	28	28	28	28	28
Renicola spp.	Pearson Correlation						0.162	0.316	−0.329	.403*	0.135	0.232
	Sig. (2-tailed)						0.409	0.101	0.087	0.033	0.492	0.234
	N						28	28	28	28	28	28
Brown Cell Inf. (DG)	Pearson Correlation							.632**	−0.275	.556**	0.371	0.296
	Sig. (2-tailed)							0.000	0.157	0.002	0.052	0.127
	N							28	28	28	28	28
Brown Cell Inf. (G)	Pearson Correlation								−0.087	.416*	0.021	.379*
	Sig. (2-tailed)								0.661	0.028	0.914	0.046
	N								28	28	28	28
Atresia	Pearson Correlation									−0.218	−0.003	−0.181
	Sig. (2-tailed)									0.265	0.987	0.356
	N									28	28	28
Granulocytoma	Pearson Correlation										0.359	.471*
	Sig. (2-tailed)										0.060	0.011
	N										28	28
Haem. Inf. (M)	Pearson Correlation											0.259
	Sig. (2-tailed)											0.184
	N											28

histopathological lesions are regularly linked to exposure to pollutants and these pathologies can affect biomarker responsiveness (Bignell et al., 2011; Garmendia et al., 2011). In many cases, the aetiology of histopathological lesions is not related to pollutants but to other factors such as parasitism (Cuevas et al., 2015). Thus, the clarification of the causality and the potential pathogenicity are of the uttermost importance in order to interpret the aetology of the histopathological lesions correctly and avoid blaming them to chemical insult.

Reproductive status is considered one the most important confounding factors when trying to assess the health status of a mussel population after applying biomarker approaches (Beyer et al., 2017; Cuevas et al., 2015; Blanco-Rayón et al., 2020). The spawning and post-spawning gonadal stages of mussels sampled in the present study, in general, belong to a late summer spawning which could correspond to the second spawning process of the year, in agreement with previous findings in mussels from the Northern Atlantic and Arctic Oceans (Duinker et al., 2008; Fokina et al., 2018; Storhaug et al., 2019). The differences observed in the gamete developmental stages might be related to different growth rates along a latitudinal axis in the study area. For instance, it has been reported that southern mussels present faster growing rates (Handå et al., 2011), and therefore it is plausible that small mussels from Trondheim are younger mussels unable to perform a second spawning in the same year in an efficient way. At the same time mussels sampled in northern latitudes seem to be older enough to perform a second spawning process (Handå et al., 2011). Moreover, part of the differences recorded in gamete developmental stages could be caused by the fact that samplings were performed sequentially within a month. Apart of size (or age), other factors such as pollution events or chronic releases of organic contaminants can trigger spawning processes due to an improved trophic condition (Dumas et al., 2020; Preisner et al., 2021), explaining the more advanced gonadal stages that were observed in mussels sampled in the vicinity of a WWTP in Trondheim. In any case, the use of mussel shell length as a proxy of age when trying to compare mussel populations with very different ecological conditions and latitudes, could lead to wrong interpretations assuming same life stages (Izagirre et al., 2014).

The Bucephalidae life-cycles are fairly uniform: the sporocysts and cercariae invariably occur in bivalves, the metacercariae in teleost fishes and the adults in predatory fishes (Lauckner, 1983). The first intermediate for hosts for *Renicola* spp. are snails (e.g. *Littorina* spp.) while the final hosts are gulls and waders, whereas the first intermediate host *G. bursicola* is unknown and in the final hosts are common eiders *Somateria mollissima* (Galaktionov et al., 2015).

Presently, the prevalence of sporocysts of trematodes (Bucephalidae)

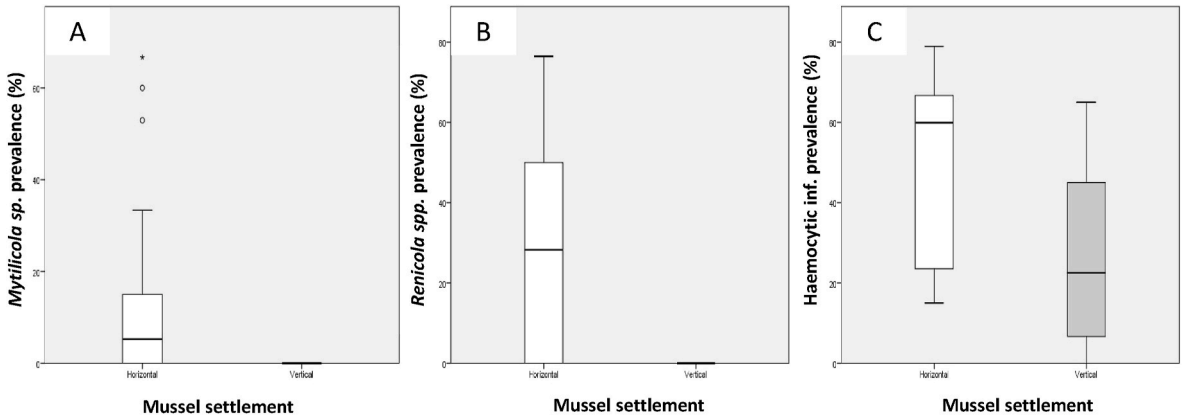

Fig. 5. Changes in distribution of the prevalence (%) of Mytilicola sp. (A), Renicola spp. trematodes (B) and haemocytic infiltration (C) in digestive gland of mussels. Differences are statistically significant ($p < 0.05$). DG: Digestive Gland.

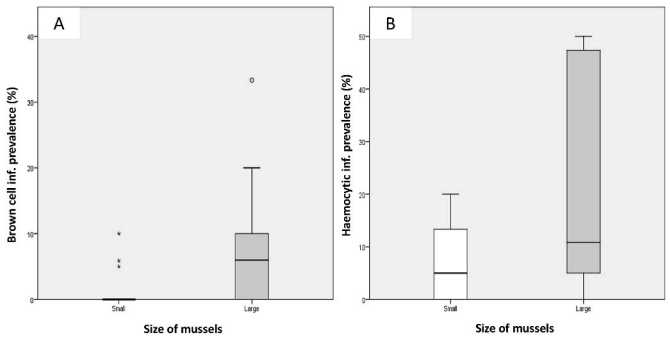

Fig. 6. Changes in distribution of the prevalence (%) of brown cell infiltration in gonad (A) and haemocytic infiltration in mantle tissue (B). Differences are statistically significant ($p < 0.05$).

were relatively low, although the infection intensity ranged from medium to high, since the infestation reached to different organs such as digestive gland, gills and gonad. In contrast, *Renicola* spp. trematodes were widespread in all latitudes along the northern Atlantic Ocean and also in Tromsø. Nevertheless, an immune reaction of the host against the parasite was appreciated in most of the cases. Moreover, significant correlations were detected between trematode metacercariae prevalence and that of granulocytomas (clearly seen in mussels from Germany). The presence of trematodes might be causing important inflammatory lesions or the presence of granulocytomas could be indicative of an immune system already battling other pathogens, such as *Francisella halioticida* (as discussed below), potentially allowing an increased infection rate of *Renicola* spp. trematode's, however these speculations require further research. Lastly, *Gymnophallus* cf. *bursicola* trematodes were only found in Iceland and Tromsø, which could be related to the distribution/presence of a final avian host (Galaktionov et al., 2015; Goater, 1993) that limited the presence of the parasite in other sampling sites. Interestingly, although the presence of a Gymnophallidae family trematode has previously been reported in mussels sampled in Connecticut (Galimany et al., 2008) with associated inflammatory responses and even with the presence of pearls, the description of the specific histological alteration produced by this parasite was not given. One of the main observed characteristics of the

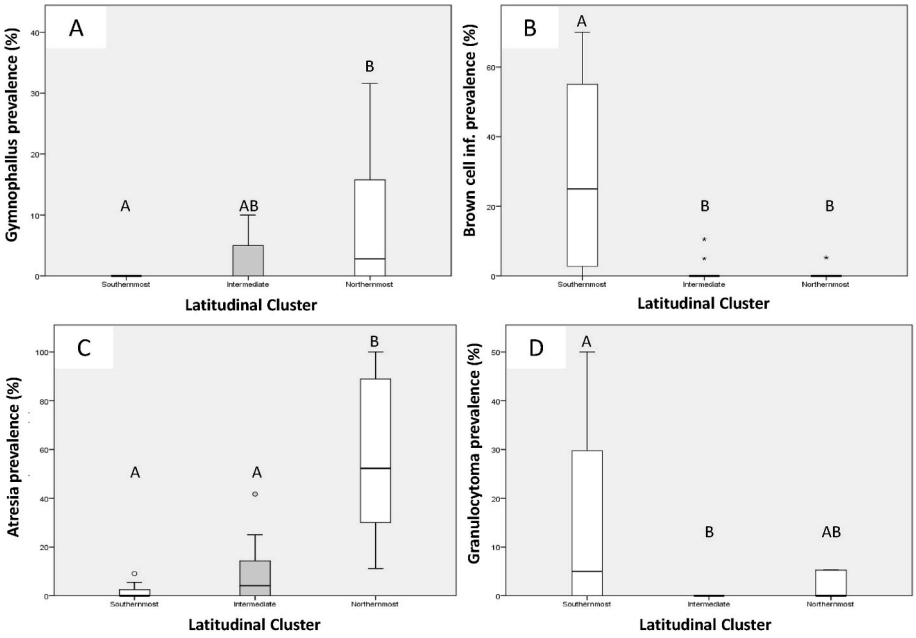

Fig. 7. Changes in the prevalence (%) of Gymnophallus cf. bursicola trematodes (A), brown cell infiltration in digestive gland (B), atresia of oocytes (C) and granulocytomas (D). Letters determine statistically significant differences ($p < 0.05$).

responses elicited by the trematode metacercariae is the appearance of pearls (Lutz, 2009). In the present study pearls were detected during the dissection and sectioning of histological samples from mussels infected with the trematode metacercariae (data not shown). Similar host reactions against *Gymnophallus* cf. *bursicola* parasites were reviewed by Lauckner in 1983 in *Cerastoderma edule*. They observed that recently attached *Gymnophallus minutus* metacercariae was surrounded by columnar epithelium cells from the pallial line and once the trematode was totally enclosed by the epithelium, it started lysing the parasite. Similarly, in the present work, cellular debris is also observed together with the presence of haemocytes in the surrounding tissues. Thus, the response elicited by *Gymnophallus* cf. *bursicola* trematode is, as far as we know, the first thorough description in *Mytilus* species. Mussels showing *Gymnophallus* cf. *bursicola* trematodes, often presented pathologies in the mantle such as atresia of the oocytes and haemocytic infiltrations in the gonad follicles or surrounding connective tissue. Pearson's test confirmed the correlation between the prevalence of the parasite and that of the haemocytic infiltration in mantle tissues. Thus, the presence of *Gymnophallus* cf. *bursicola* trematodes as intermediate hosts in mussels is worth to take into account in health status assessment of mussels, which could have implications in marine pollution monitoring. In fact, biomarker studies conducted in 2016 in mussels from the same sampling site in Tromsø that presented *Gymnophallus* cf. *bursicola* trematodes and in biomarker studies conducted in mussels collected in the same sampling campaign as in the present work stress responses that could only be linked to the presence of *Gymnophallus* cf. *bursicola* trematodes have been reported (Benito, 2022).

The second type of metazoan parasites found were the *Mytilicola* sp. copepods. The pathogenicity is considered to be low although under certain circumstances (low food availability, spawning processes …) the condition index of mussels can be affected (Pérez Camacho et al., 1997). In the present study no important pathogenic effect was detected in relation to *Mytilicola* sp. infection. However, the presence of this parasite is conditioned by the sampling site characteristics and correlated with *Renicola* spp. trematodes prevalence, as it will be discussed below.

The prevalence and infection intensity of IMB was relatively low and similar to previous reports. IMB are common parasites in mussels and although they can cause hypertrophy and degeneration of the infected cells that can be fatal (Garmendia et al., 2011). The results could suggest that the impact on the present mussels populations is low since no histological alterations that could indicate pathogenicity were detected.

As far as IC parasites are concerned, it seems that other studies have described similar pathogens as Multinucleate Parasite X (MPX) (Fichi et al., 2018). The infection prevalence was in line with what it has been described in the U.K. in previous studies, (Fichi et al., 2018), while the infection intensity was in every case below 50 parasites per slide, what it has previously been considered as a moderate infection (Gombac et al., 2008). No host response was detected in mussels infected with intracellular ciliates, so the harmfulness of this parasite seems to be low or non-existent in the present work, which is concordant with previous studies (Fichi et al., 2018; Gombac et al., 2008; Villalba et al., 1997).

The histological analyses of the mussel gonads found three evident pathologies: brown cell infiltration in gonad follicles, atresia of the oocytes and haemocytic infiltration in mantle tissues. The presence of brown cells in gonad follicles and atresia of oocytes are indicators of ongoing autolysis and resorption processes during gametogenesis, and can be induced when environmental conditions become unfavorable for spawning or when energy is required under stressing conditions after gamete maturation (Smolarz et al., 2017; Suárez et al., 2005). Thus, these alterations were expected to occur more commonly in individuals with a more advanced gametogenic state (Cuevas et al., 2015) as in the case of mussels from Svalbard. Brown cell and haemocytic infiltration in gonad follicles and surrounding tissues were commonly found in groups where the sum of mussels in spawning and post-spawning stages was high and it were more likely to be related with the resorption of the remaining gametes and reproductive tissue. The histological analyses of the digestive gland found the following inflammatory responses: brown cell infiltration in digestive cells, haemocytic infiltration in digestive or connective tissue (also in gills) and the presence of granulocytomas. Brown cell infiltration in digestive tissue was probably related to the recovery process after inflammatory episodes (Bignell et al., 2011), and the presence of brown cells in male gonad follicles could also be indicative of some kind of distress causing the gametes to be reabsorbed, as discussed above. The fact that the prevalence of brown cell infiltration in digestive tissue was correlated with the prevalence of brown cell infiltration in gonad and with granulocytoma might indicate an ongoing systemic immune response. Moreover, the granulocytoma prevalence is also correlated to haemocytic infiltration in digestive gland, which could indicate a worrying pathological status in those mussel groups with the highest prevalence (large mussels from the clean sites in Germany and Scotland, and from the impacted site in Scotland). Haemocytic infiltrations are often related to the presence of pollutants, starvation processes, parasitic infection and spawning stress (Garmendia et al., 2011). Thus the high haemocytic infiltration prevalence found in mussels from different sites should be taken into account as the aetiology of the lesion can be completely different (Cuevas et al., 2015). Referring to the characteristics of the sampling sites and the presence of concurrent parasites and pathologies, in the present study the haemocytic infiltration cases might have been caused by anthropogenic impact (small and large mussels from the impacted sites in Oslo and Iceland) or by parasitism (mussels from the WWTP in Trondheim and mussels from the clean site in Iceland), although other physiological and environmental causes cannot be discarded. The case of mussels from Scotland and Germany seems specially worrying since the high prevalence of haemocytic infiltration is accompanied (at least in large mussels) by a high presence of granulocytomas which are inflammatory responses resulting in vascular occlusions that have been linked to chronic stress (Lowe and Moore, 1979). A similar pathological status has been described in mussel beds or cultures that have suffered from high mortality episodes, like the ones described in the Netherlands in 2015/16 and 2019 (Capelle et al., 2021) where up to 70% of the mussels presented one or multiple granulocytomas. Similar episodes have been described in France since 2014 (Charles et al., 2020a). Although the cause of these episodes is unclear, the presence of a bacteria responsible for previous mortality episodes in other bivalves (*Francisella halioticida*) has been confirmed in granulocytomas found in mussel populations suffering from mortality episodes in France (Charles et al., 2020b) and also in mussels from the UK (Cano et al., 2021). Although the severe histopathological condition presented by mussels from Scotland and Germany might be caused by many unknown factors, the similarities with previously described mortality episodes are to be taken into account. Thus, further research and monitoring are necessary to assess the cause, the possible geographical extension and the temporal evolution of these events. Indeed, in Scotland loss of wild mussel beds were observed in late 2017 (personal communication by Dr. Stefano Carboni).

The fact that no differences in the prevalence of pathologies and parasitism were found when clustering all the groups by level of anthropogenic impact indicates that in the present study this factor seems not be the main one rendering differences in the histopathological profile of mussel populations. Previous studies (Cuevas et al., 2015) defined the use of a histopathological index that is responsive to the level of pollutants in the environment and although a minimum confounding effect was caused by seasonal variability and by parasitosis the index proved to be effective. However, the use of histopathological analysis as an indicator of exposure to pollutants could cause a misinterpretation of the results given the particular structure of the present work with mussels collected from a large geographical distribution and different sizes that include mussels of very different ages. Nevertheless, the lack of statistical significance does not mean that pollution did not have an effect in any of the pathologies, since its statistical significance could have been masked by other factors. In this sense, another known confounding factor that could interfere with the assessment of the health

status of mussels is where mussels are collected from (Beyer et al., 2017). When performing studies for the biomonitoring of biological effects caused by exposure to pollutants it is hardly avoidable to sample in such different substrates. For example, the principal goal of the sampling campaign was to compare the responsiveness of biomarkers between impacted (mostly harbours) and clean sites (mostly rocky shores) along a large latitudinal span. The presence of *Mytilicola* sp. and *Renicola* spp. trematodes was significantly higher in mussels sampled in horizontal substrates, which is coherent with previous studies since it has been demonstrated that mussels collected from benthic intertidal beds present higher parasitic burden than the ones collected from ropes, pylons and similar off-shore vertical structures (Buck et al., 2005). In the cited work it is stated that one of the conditions to avoid off-shore vertical structures being a more suitable environment for parasite proliferation is to avoid fouling communities by periodical cleaning, as it would increase the presence of intermediate hosts. In the present work the reason behind the parasitic burden differences regarding horizontal/vertical substrates could be caused by the fact that the sampling sites categorized as horizontal substrates constitute a more suitable environment for initial, intermediate and final hosts. On the contrary, sampling sites where mussels were collected from vertical substrates were less suitable for other hosts necessary for the parasites to close their life cycle. In addition, the prevalence of haemocytic infiltration was also higher in mussels sampled in horizontal substrates, probably as a consequence of a higher parasitic burden in those mussels and not by the presence of pollutants as discussed before. It might be helpful avoiding additional confounding effects when planning biomonitoring programs and taking into account the characteristics of the substrate where mussel populations are sampled from. Moreover, environmental monitoring protocols might indicate that mussels of comparable shell length must be sampled (Beyer et al., 2017) as size is considered a confounding factor for the interpretation of biological responses (Blanco-Rayón et al., 2019). In the present study only pathologies related to the reproductive cycle (brown cell and haemocytic infiltration in gonad and surrounding connective tissue) displayed statistically significant differences when comparing small to large mussels, the latter presenting higher prevalence. As discussed before, these differences are probably caused by the more advance gametogenic stages in large mussels mostly in the southernmost and intermediate localities, and thus, it is reasonable to find differences regarding biological responses against pollution, so sampling mussels of comparable size seems to be an adequate guideline.

When comparing the prevalence of parasites and pathologies with the sampling groups clustered by latitudinal location, significant differences were found in the prevalence of *Gymnophallus* cf. *bursicola* trematode, which is not surprising as it was only found in Iceland and Tromsø and could be related to the distribution of its final host. Two pathologies that were significantly more present in the southernmost sampling points were granulocytomas and brown cell infiltration in digestive tissue. It seems that their prevalence was correlated and their significantly higher presence was caused by the exceptional histopathological status in mussels from Germany and Scotland as mentioned before. Atresia was significantly more prevalent in the northernmost locations and this significant difference was probably due to its high prevalence in mussels from Svalbard, which on the other hand was coherent with the fact that those were the groups with the biggest proportion of mature gametes in accordance with their most prevalent spawning stage.

5. Conclusion

The new data provided here offers a prospective view of the variability and distribution of parasites, pathologies and their effect on wild mussel populations in different environments along a wide latitudinal span along the Northern Atlantic and Arctic Oceans. A latitudinal gradient is clearly observed in gamete developmental stages as northern and southern mussels presented different conditions at the same period. Furthermore, the size/age relationship seemed not to be comparable latitudinally with evident differences in the reproductive cycle and the appearance of related pathologies. In addition, specific parasitic profiles ruled by latitudinal conditions and the settlement of mussels (horizontal/vertical) have been demonstrated to be of a great importance in the health condition of mussels. The present work also provides the first histological description of the host response *Gymnophallus* cf. *bursicola* parasite that caused considerable alterations the mantle of mussels in Tromsø and Iceland plus the report of grave histopathological status that included high prevalence of granulocytomas in Scotland and Germany. It can be concluded that it is necessary to perform a thorough histological analysis of mussels, as histopathological conditions are related to factors such as latitude, size/age, settlement and parasites that could compromise the environmental health status assessment in marine pollution monitoring.

CRediT authorship contribution statement

Denis Benito: Conceptualization, Methodology, Investigation, Writing – original draft. **Dragana Paleček:** Methodology. **Xabier Lekube:** Validation, Investigation, Writing – review & editing. **Urtzi Izagirre:** Methodology, Validation, Writing – review & editing. **Ionan Marigómez:** Validation, Project administration, Funding acquisition. **Beñat Zaldibar:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. **Manu Soto:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision.

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.

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