Science of the Total Environment 678 (2019) 207–216

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Mucous cell responses to contaminants and parasites in shorthorn sculpins (*Myoxocephalus scorpius*) from a former lead-zinc mine in West Greenland

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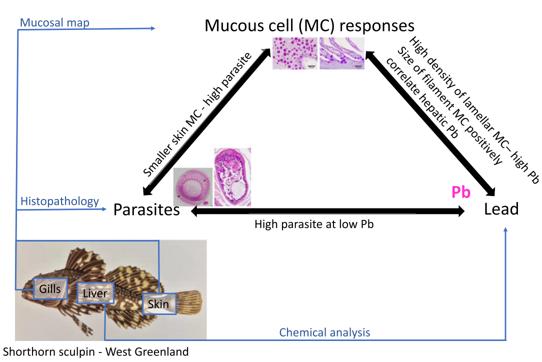
HIGHLIGHTS

- Skin and gill mucosal epithelia reacted to environmental contamination and parasites.
- Smallest skin mucous cells at the lowest Pb residue in liver and highest digenean load in skin.
- Significant effect of station on density of gill lamellar mucous cells following Pb gradient in sediments.
- Size of filament mucous cells positively correlated with Pb concentrations in liver.
- Mucosal epithelial changes can be used as biomarkers in field studies.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 February 2019
Received in revised form 21 April 2019
Accepted 27 April 2019
Available online 29 April 2019

Editor: Henner Hollert

Keywords:
Mucosal mapping
Heavy metals
Parasite
Skin
Gills
Mining

ABSTRACT

Previous studies of sculpins from the former lead (Pb) - zinc (Zn) mine near Maarmorilik, West Greenland, have shown that these fish are affected by heavy metal exposure from the mine. In this study, we applied mucosal mapping (a stereological method for mucosal quantification in fish) to uncover interactions between the host, parasites and heavy metal exposure (Pb and Zn) in shorthorn sculpins from the Maarmorilik mining site at a gradient of 3 stations. Skin and gill mucosal epithelia of shorthorn sculpins were significantly affected and reflected the exposure to environmental heavy metals and parasites. Size of skin mucous cells was significantly smallest in the sculpin from the station 3 where heavy metal contamination was lowest and the skin parasite load was highest. Gill filament mucous cells were largest and densest in fish from station 1 which was the most contaminated site. In gill lamellae the density of mucous cell followed a toxicity gradient and was significantly highest at the most contaminated station and significantly lowest at the least contaminated station. The persistent presence of toxic Pb and Zn levels in the sediment at the most contaminated station may have induced a small but measurable reduction in the surface area available for respiration and may have affected diffusion distance. The strong correlation between size of filamentous mucous cells and Pb concentrations in liver suggests that these cells can

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https://doi.org/10.1016/j.scitotenv.2019.04.412

play an active role in reducing the somatic load of Pb in sculpin. We suggest that mucosal mapping can be used to assess effects of contaminant and parasite exposure in future environmental field studies.

1. Introduction

Aquatic pollution has been a global concern because most waterways are, to some extent, contaminated which can subsequently cause human health problems (Ansari et al., 2004; Islam and Tanaka, 2004; Rheinberger and Hammitt, 2012). Waterways are polluted by a number of contaminants such as fertilizers, pesticides, agrochemicals, domestic waste, sewage sludge, oils, heavy metals, trace elements, organic compounds, plastics, sediments, nutrients and biological pollution (Islam and Tanaka, 2004). Heavy metals are common anthropogenic contaminants in coastal areas and marine waters worldwide and elevated concentrations of these elements in the environment can be toxic to aquatic organisms (Ansari et al., 2004; Govind, 2014; Naser, 2013).

Contamination of the water and sediment around Maarmorilik, West Greenland with elements related to the former Black Angel lead (Pb) and zinc (Zn) mine is well-documented (Johansen et al., 2008; Søndergaard et al., 2011; Sonne et al., 2014). During the operation period (1973–1990), the mine discharged several million tons of tailings and waste rock into the fiord and on nearby mountain slopes causing dispersion of heavy metals including Pb, Zn and cadmium (Cd) (Johansen et al., 2008; Perner et al., 2010). Concentrations of dissolved metals in sea water near the mine peaked during the active mining period (Pb: 440 μg/L, Zn: 790 μg/L) and were reduced abruptly after closure (Søndergaard et al., 2011). By 2008, the concentrations of dissolved Pb in the water were reduced to 1/1000 of peak contamination levels and dissolved Zn to 1/10 of peak levels (Søndergaard et al., 2011). Nonetheless, Pb and Zn in bottom sediment near the mine remained high. In 2017, only moderately elevated levels of dissolved Pb and Zn in water were detected near the mine whereas levels of these metals in sediment were still at concentrations considered potentially highly toxic (Hansson et al., unpublished results).

In Maarmorilik, high levels of heavy metals (especially Pb and Zn) in the water and sediments are reflected in the bioaccumulation in marine biota including seaweed, mussels, sea snails and fish such as shorthorn sculpins (*Myoxocephalus scorpius*) (Johansen et al., 2008; Søndergaard et al., 2011, 2014). The concentrations of Pb in liver samples of shorthorn sculpins caught near the mine were significantly higher than those collected up to 12 km away (Søndergaard et al., 2015; Sonne et al., 2014). However, the concentrations of Zn in the sculpin livers did not show a significant variation across stations (Sonne et al., 2014). This variation in concentration patterns of elements in sculpins may be due to differences in biological need, uptake, metabolism and excretion of these elements (Wood et al., 2011a, 2011b)

Pb is a non-essential element with no biological function (Mager, 2011). Pb is taken up mainly via the mucosal epithelial barriers of gills, gut, and skin (Macdonald et al., 2002; Mager, 2011; Rogers, 2004). Fish have several routes to excrete Pb including the gills, kidney, liver and gut (Mager, 2011). There is evidence that fish excrete Pb in mucus in both gills and gut (Varanasi and Markey, 1978). The sloughing of mucus represents the first response to exposure to Pb (Ojo and Wood, 2007). In contrast to Pb, Zn is an essential element required for multiple biological processes such as active proliferation and differentiation of epidermal cells, stimulating re-epithelialization, modulating inflammation, stimulating wound healing and removal of cells afflicted by necrosis (El-Adl et al., 2018; Fosmire, 1990; Hogstrand, 2011; Jensen et al., 2015). In marine fish, Zn uptake occurs mainly through gut and gills (Hogstrand, 2011). Some evidence of interactions between mucous cells responses and Zn uptake has been reported in the gut of rainbow trout but not in the gills (Hogstrand, 2011). The excretion of Zn occurs via gills, liver, kidney, intestine and oocytes and may possibly not relate to mucus excretion (Hogstrand, 2011).

Mucosal barriers including skin, gills and gut are essential to ecotoxicological monitoring of fish (Bols et al., 2001; McKim and Lien, 2001; Vatsos et al., 2010; Wood, 2001). Mucosal epitheliums are the first immune barrier that confront environmental challenges such as pollutants and parasites and they respond constantly to these challenges (Gomez et al., 2013; Jensen, 2015; Schlenk and Benson, 2001). Fish exposed to sub-lethal chemical stressors such as heavy metals, release large amounts of mucus to reinforce protective barriers, which delays the diffusion of chemicals (Bols et al., 2001; McKim and Lien, 2001; Wu et al., 2007). The mucus is able to bind organic and inorganic materials and to remove them with constant secretion to the environment. As a result, chronic exposure of the mucosal epithelial layer to toxins results in fewer mucous cells and thinner epithelium (McKim and Lien, 2001). When the immune response is enhanced, mucosal responses display a reduction in size of mucous cells while the density remains the same (Torrecillas et al., 2015). When faced with parasitic challenges, fish commonly react with an increased secretion of mucus. Skin and gill parasites, for example trichodinids, feed on mucins (Lom and Dyková, 1992).

Shorthorn sculpin is a heavily parasitized species (Dang et al., 2017; Nørregaard et al., 2018; Scott and Scott, 1988) and sculpins caught in contaminated areas in Maarmorilik are well documented as being affected by heavy metal contaminants (Johansen et al., 2008; Søndergaard et al., 2011; Sonne et al., 2014). To understand the interactions among parasites, pollutants and host mucosal responses, it is critical to properly quantify mucous cell populations in barrier tissues like skin and gill epithelia. Mucosal mapping method is a novel, quick and efficient method for stereological quantifying mucosal responses in fish (Pittman et al., 2011, 2013a, 2013b; Torrecillas et al., 2015). In this study, we applied mucosal mapping to shorthorn sculpins caught near the former Pb—Zn mine in Maarmorilik, West Greenland, to uncover interactions between the host, parasites and element exposure (Pb and Zn).

2. Material and methods

2.1. Study area

Three stations representing different levels of Pb and Zn contamination were selected for this study (Fig. 1). These stations were located along a distance gradient from the Black Angel mine in Maarmorilik, West Greenland. Fish and environmental samples were collected in August 2017.

Station 1 was located near the mine and affected by leaching of heavy metals from tailings, waste rock and other remains from the mine resulting in significant Pb and Zn contamination (Johansen et al., 2008; Søndergaard et al., 2011, 2014; Sonne et al., 2014). In 2017, levels of Pb in sediments were 22 and levels of and Zn were 6 fold greater than Probable Effect Levels (PELs) for these elements (CCME, 2001) (Table 1). Concentrations of investigated elements (chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), Zn, silver (Ag), cadmium (Cd) and Pb) in seawater were below guideline levels for protection of aquatic life (Government of Greenland, 2015; Howe et al., 2004; Nagpal et al., 2004; Simpson et al., 2013).

Station 2 was located 5 km west of the mine and influenced by the mine with lower levels of Pb but highest level of Zn. Station 3 was situated 12 km from the mine and was considered unaffected or minimally

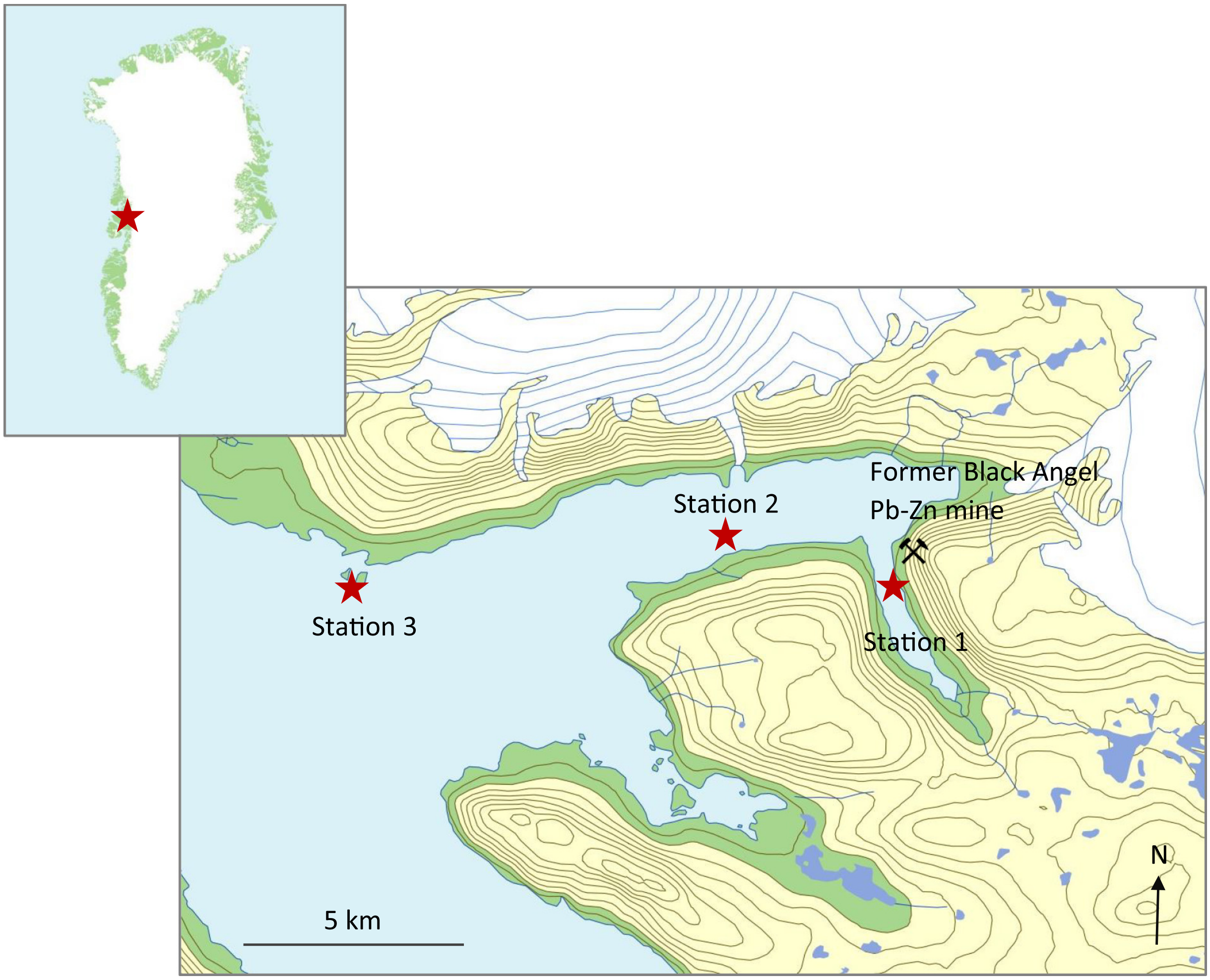

Fig. 1. Sampling stations near the former Black Angel lead-zinc mine in Maarmorilik, West Greenland. Station 1 (71°7′3.37″ N; 51°15′6.15″ W); station 2 (71°7′13.19″ N; 51°21′29.14″ W); and station 3 (71°5′57.57″ N; 51°34′14.03″ W).

Table 1
Metal levels in sculpin liver (μg g^{-1} wet weight (w.w.)), sediment (μg g^{-1}) and seawater (μg/ L labile metal as measured by DGT) among three sampling stations. Red: above Probable effect levels (PELs); Green: below PELs or at background levels. Adapted from [a]: CCME, 2001; [b]: Howe et al., 2004; [c]: Nagpal et al., 2004; [d]: Simpson et al., 2013; [e]: Government of Greenland, 2015; [1]: 'background levels' (reported in several uncontaminated marine stations in Europe, Canada and Australia) were used as there was no available guideline for these elements. *: reference values do not specify whether labile or total concentrations are used.

<d.l.: lower than detection limit

Liver	Site	n		Cr	Mn	Co	Ni	Zn	Ag	Cd	Pb
	1	5	Mean	<d.l.	0.52	0.04	<d.l.	17.7	0.02	0.28	0.14
			SE		0.08	0.01		1.21	0.01	0.08	0.06
	2	5	Mean	<d.l.	0.55	0.04	<d.l	24.9	0.02	0.42	0.04
			SE		0.11	0.02		3.00	0.01	0.21	0.01
	3	5	Mean	<d.l.	0.40	0.04	<d.l.	22.4	0.04	0.32	0.02
			SE		0.04	0.01		3.21	0.03	0.19	0.00

Supporting data

Sediment	Site	n	Cr	Mn	Co	Ni	Zn	Ag	Cd	Pb
Probable effect levels			160[a]	800[1b]	24.5[1c]	52[d]	271[a]	4[d]	10[a]	112[a]
	1	1	61.8	600	13.1	39.1	1509	1.33	5.19	2497.6
	2	1	57.5	418	10.4	36.1	161	0.03	0.47	73.8
	3	1	40.6	299	6.3	22.1	58	0.01	0.15	15.3

Seawater	Site	n	Cr	Mn	Co	Ni	Zn	Ag	Cd	Pb
Water quality guidelines			3[e]	200[b*]	0.3[1c*]	5[e]	10[e]	0.1 [d*]	0.2[e]	2[e]
	1	1	<d.l.	0.54	<d.l.	0.21	0.85	<d.l.	0.02	0.11
	2	1	<d.l.	0.41	<d.l.	0.24	1.48	<d.l.	0.02	0.06
	3	1	<d.l.	0.40	<d.l.	0.30	0.54	<d.l.	0.01	0.02

affected by the mining pollution (Søndergaard et al., 2011; Sonne et al., 2014). In 2017, Pb and Zn in water and sediment were at background levels in station 3 and at or just above background level in station 2 (CCME, 2001; Government of Greenland, 2015) (Table 1, (Hansson et al., unpublished results).

2.2. Sampling

Thirty shorthorn sculpins (*Myoxocephalus scorpius*) were collected and handled at the three stations according to permission granted by the Greenland Government to Jens Søndergaard and Lis Bach (project no 771020). After being gently removed from the fishing hook, the fish were kept alive in 20 L buckets with seawater until dissection. Water was renewed regularly to make sure that temperature and oxygen levels remained favourable for fish survival.

Table 2
Fish biometric (mean ± SE) of shorthorn sculpin from each station. * indicates a significant difference between male and female.

Site	Sex	n	Weight*	Length*	Liver weight*
			g	cm	g
1	Female	7	697 ± 90	36.8 ± 1.5	70.9 ± 13.2
	Male	3	354 ± 50	29.7 ± 1.7	29.0 ± 6.1
2	Female	7	506 ± 40	34.3 ± 1.0	43.0 ± 9.5
	Male	3	305 ± 74	31.4 ± 4.0	26.0 ± 9.0
3	Female	7	560 ± 40	35.3 ± 0.8	43.9 ± 2.6
	Male	3	258 ± 7	27.0 ± 0.8	19.3 ± 3.3

After landing, total body weight (g) and length (cm) were measured and recorded for each fish (Table 2). Collection of samples for mucosal mapping was conducted with minimum fish handling (i.e. particular care was taken so that the area for collection was not touched). A skin sample from the tail area and the second left gill arch were collected using scalpel and tweezers without touching the surface area or the soft tissue, then carefully placed into labelled histocassettes and fixed in 4% phosphate buffered formalin. The fish then were dissected and sex was recorded. In every individual fish the same piece of the liver was collected and placed into pre-marked plastic zip lock bags and frozen until subsequent chemical analyses.

2.3. Sample processing and data collection

2.3.1. Mucosal samples

Mucosal epithelia were processed using routine histology with the exception of tangential embedding and sectioning rather than transverse, as described by Pittman et al. (2011) (Fig. 2A–B). 3 μm sections were stained with Periodic Acid Schiff/Alcian Blue (PAS/AB) for visualization of mucous cells in skin and gills (Fig. 2D–F). Mucous samples were analysed at the Quantidoc lab at the Department of Biology, University of Bergen, Norway, January 2018, using the method described by Pittman et al. (2011, 2013b). Briefly, sections were analysed using a Leica Axioskop microscope combined with a Prior Proscan digital stage and the Visiopharm Integrator System (VIS). At least 100 cells were measured (Pittman et al., 2013b). Mucous cell number, size and volumetric density in epithelia of skin, gill filament and gill lamellae gave three mucosal indexes:

Mucous cell area (1) was the mean size of mucous cells in that individual's tissue.

Mucous cell density or % epithelium filled with mucous cells (2)

$$= \frac{\text{Mucous cell area} \times \text{mucous cell number}}{\text{Epithelial area}} \times 100$$

$$\text{Barrier status (3)} = \frac{1}{\text{Mucous cell area}/\text{Mucous cell density}} \times 1000$$

The same paraffin blocks of mucosal samples (the gills and skin) were tangentially sectioned at 3 μm and stained with Haematoxylin and Eosin (HE) to determine presence of parasitic infections. Parasites on the gills were identified from histology. Morphology of the parasites (integument and attachment organs) in the skin was used for identification (Bruno et al., 2006). Quantification of parasites was done using intensity criteria achieved from interpretation of histological sections of gills and skin. Briefly, intensity of parasites in skin was estimated by number of parasites per cm^2 of surface area of histological sections. Surface areas were measured using Image J software after calibration. Intensity of gill parasites was the number of individuals per filament.

2.3.2. Liver samples

For each station, livers from 5 fish (total n = 15) were sampled to analyse for liver metal content following the protocol described in Sonne et al. (2014). Briefly, 1 g frozen tissue was subsampled from each liver and digested using 4 mL Merck Suprapur HNO_3 and 4 mL mQ H_2O in teflon bombs under pressure in a microwave oven (Anton Paar Multiwave 3000). After digestion, all samples were transferred to polyethylene bottles and diluted with mQ H_2O to reach a final solution of 60 mL, which was analysed for geochemistry (Cr, Mn, Co, Ni, Zn, Ag, Cd, and Pb) using an Agilent 7900 ICP-MS at the trace element laboratory in Roskilde, Denmark. The data are shown in Table 1 and is reported in μg g^{-1} wet weight (w.w.). Analytical quality control was verified by including blanks, duplicates and certified reference materials (DOLT-4,

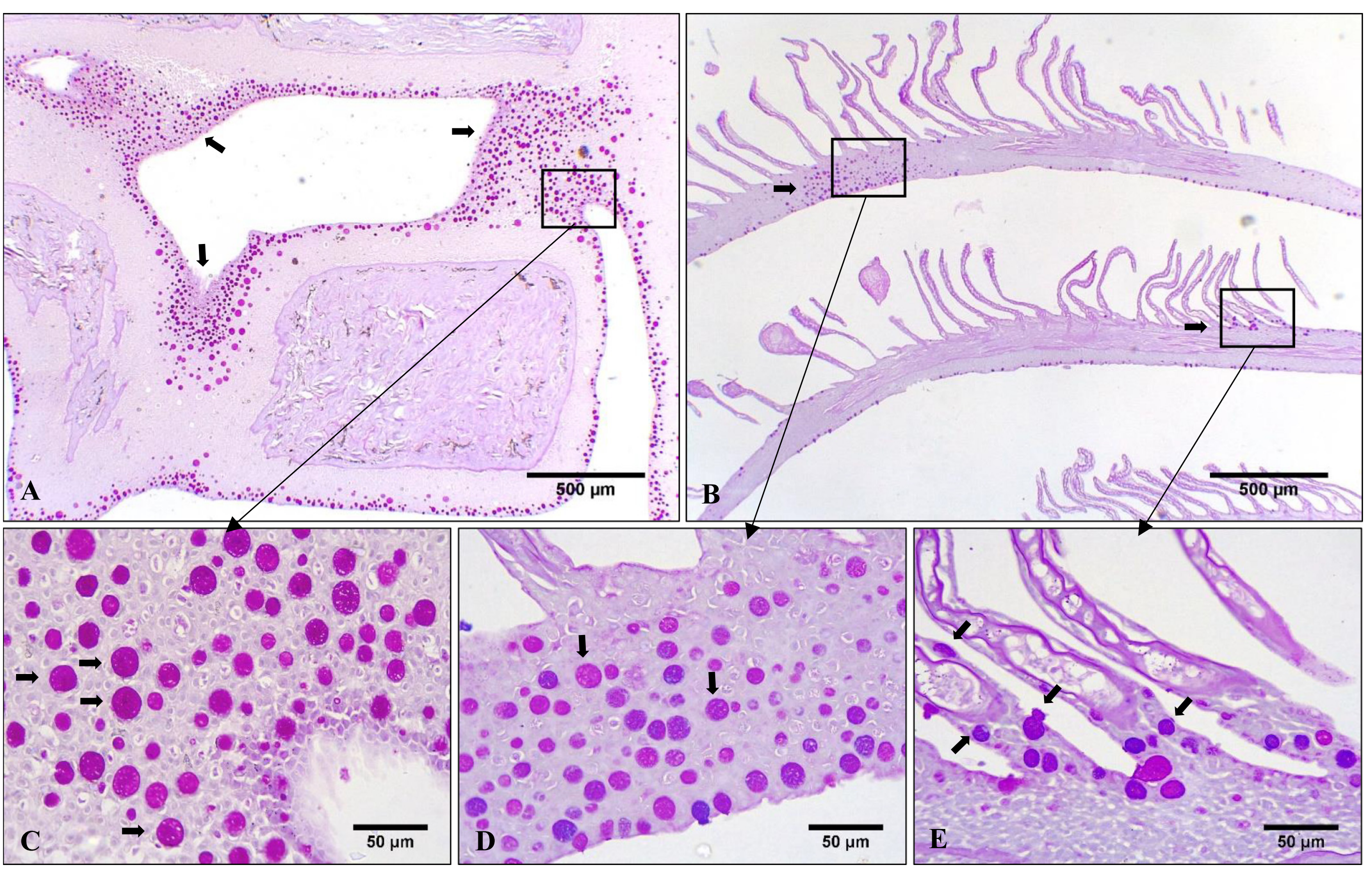

Fig. 2. Tangential sections of skin and gills of shorthorn sculpins from Maarmorilik, West Greenland were used for mucosal mapping (PAS/AB stain). A. A section of skin showing epithelial layer filled with mucous cells (arrows). B. Superficial layer of gills showing a number of mucous cells (arrows). C. Skin mucous cells (arrows). D. Filament mucous cells (arrows). E. Lamellar mucous cells (arrows).

DORM-4, TORT-2, MESS-4 and PACS-2) in the analyses, and detailed information can be found in the supplementary information (Table S1).

2.4. Data analyses

Biometrics (body length, body weight and liver weight), level of metals in fish organs, mucous cell responses (mucous cell area, density and barrier status indexes) and parasite intensity were averaged and presented as mean ± standard error (SE) for each sampling site. Because only 5 liver samples were analysed for metal levels at each station, all statistical tests using hepatic metal data (including variation in hepatic metals among stations, correlation between hepatic metals and mucous cells responses or parasite infection) were based on 15 individuals whereas the rest of statistical analyses used the data from the total 30 fish. The potential effect of station on each mucosal index was investigated using a oneway ANOVA (IBM SPSS statistic 22). Gender differences in pattern of mucosal responses were assessed by comparing males and females using an independent *t*-test (un-equal sample size, IBM SPSS statistic 22). A Levene test and histogram were used to check for assumption of equal variances and normal distribution prior to oneway ANOVA and independent t-test. Log transformation of data was made when the assumptions were violated.

Associations between mucosal responses and metal accumulation level, biometrics or parasite intensity of individual fish from all locations were assessed using Pearson correlation analysis (IBM SPSS statistic 22). Level of significance was $P < 0.05$. A Chi-square of independence was used to check for differences in prevalence of parasite infections among stations.

3. Results

3.1. Fish biometric and sex ratio

There was no significant difference in body length (33.6 ± 0.8 cm), body weight (503.2 ± 36.7 g) or liver weight (44.2 ± 4.9 g) for sculpins between the 3 sites. Sex ratio was evenly distributed among group with 7 females: 3 males in each group (Table 2).

Body length was positively correlated with body weight ($R = 0.87$, $n = 30$, $P < 0.001$) and liver weight ($R = 0.81$, $n = 30$, $P < 0.001$). Body weight also correlated well with liver weight ($R = 0.88$, $n = 30$, $P < 0.001$) as expected.

3.2. Metal levels in liver

There were only small variations between stations in most of the investigated elements (Cr, Mn, Co, Ni, Ag and Cd). However, the average Pb concentration in liver of fish caught at Station 1 (0.14 μg g^{-1} w.w.) was about 3.5 fold higher than those at station 2 (0.04 μg g^{-1} w.w.) and 7 fold greater than those at station 3 (0.02 μg g^{-1} w.w., Table 1). Average levels of Zn in livers were lowest at station 1 (17.7 μg g^{-1} w.w.) and highest at station 2 (24.9 μg g^{-1} w.w.).

Significantly positive correlations were found between the levels of liver Ag, Mn and Cd with body length/weight and liver weight of shorthorn sculpin of both genders (Table 3). When separating into sex, moderate correlations were detected in males between liver Pb and biometric parameter including body and liver weight (Table 3). In females, length was positively correlated with hepatic levels of Mn, Co, Ni, Ag and Cd. A strong positive correlation between weight of female and level of Pb in the liver was evident (Table 3).

3.3. Parasite load

In this study, the sampled fish were heavily infected by a number of parasite species including the digeneans primarily in the skin and trichodinids on the gills.

Metacercariae were observed in the dermis and the underlying muscle of shorthorn sculpin (Fig. 3A–B) despite only a small area (about 1cm^2) being examined. The prevalence of infection of fish from station 3 (50%) was higher than in other two stations (10–30%) (Fig. 4A). Sculpins at station 3 were more infected by this parasite (2.1 ± 1.1 digenea/cm^2) than those in stations 1 and 2 (0.5 ± 0.3 and 0.2 ± 0.1 digenea/cm^2, respectively). The maximum intensity of 10 digenea/cm^2 was observed in a single fish from station 2 (Fig. 4B).

Trichodinids were commonly found in histological sections of the gills of shorthorn sculpin (Fig. 3C–D) with their prevalence reaching 70% in station 1, 60% in station 2 and 90% in station 3 (Fig. 4A). The number of trichodinids per filament varied from 4.76 ± 3.22 (station 1) and 5.15 ± 2.66 (station 2) to 8.66 ± 2.86 (station 3) again following the inverse environmental gradient (Fig. 4B). In a heavily infected case, 33.19 trichodinids were observed per gill filament of shorthorn sculpin.

3.4. Variation of mucosal responses among stations

3.4.1. Skin

On average, mucous cells on the skin of fish from station 3 were only 113.38 ± 13.09 μm^2 in size whereas those from station 1 and 2 were from 166.69 ± 30.34 to 186.49 ± 26.26 μm^2. The size of skin mucous cells from station 3, where Pb and Zn were at lowest level, was significantly smaller than those from station 2 ($P = 0.017$, Fig. 5A). No difference was detected in skin mucous cell density, which varied around 4% of the epithelial volume ($P = 0.469$, Fig. 5B), among the 3

Table 3
Correlation between metal levels in the liver and biometric parameters of shorthorn sculpin from all sampled locations. * significant difference at $P < 0.05$; ** significant difference at $P < 0.01$.

			Cr	Mn	Co	Ni	Zn	Ag	Cd	Pb
Both sex (n = 15)	Length	R	−0.115	0.637*	0.303	−0.139	0.393	0.493	0.605*	0.054
		p	0.682	0.011	0.273	0.621	0.147	0.062	0.017	0.847
	Weight	R	−0.039	0.263	−0.097	−0.167	0.086	0.564*	0.303	0.012
		p	0.891	0.343	0.732	0.551	0.760	0.029	0.272	0.967
	Liver weight	R	0.003	0.438	−0.090	−0.217	−0.133	0.208	0.162	0.032
		p	0.991	0.102	0.750	0.437	0.637	0.458	0.563	0.911
Male (n = 6)	Length	R	0.240	0.305	−0.021	−0.507	0.071	0.582	0.589	0.620
		p	0.534	0.425	0.958	0.164	0.856	0.100	0.095	0.075
	Weight	R	0.372	0.327	0.036	−0.420	0.113	0.629	0.628	0.710*
		p	0.324	0.390	0.927	0.261	0.773	0.070	0.070	0.032
	Liver weight	R	0.342	0.573	−0.231	−0.376	−0.431	0.137	0.136	0.798**
		p	0.367	0.106	0.550	0.319	0.247	0.726	0.727	0.010
Female (n = 9)	Length	R	−0.248	0.944**	0.893*	0.833*	0.682	0.823*	0.937**	0.442
		p	0.636	0.005	0.017	0.039	0.135	0.044	0.006	0.381
	Weight	R	−0.569	0.306	0.119	0.018	−0.259	0.582	0.260	0.941**
		p	0.239	0.555	0.822	0.973	0.620	0.226	0.618	0.005
	Liver weight	R	−0.618	0.724	0.741	0.661	0.408	0.618	0.751	0.492
		p	0.191	0.104	0.092	0.153	0.422	0.191	0.085	0.322

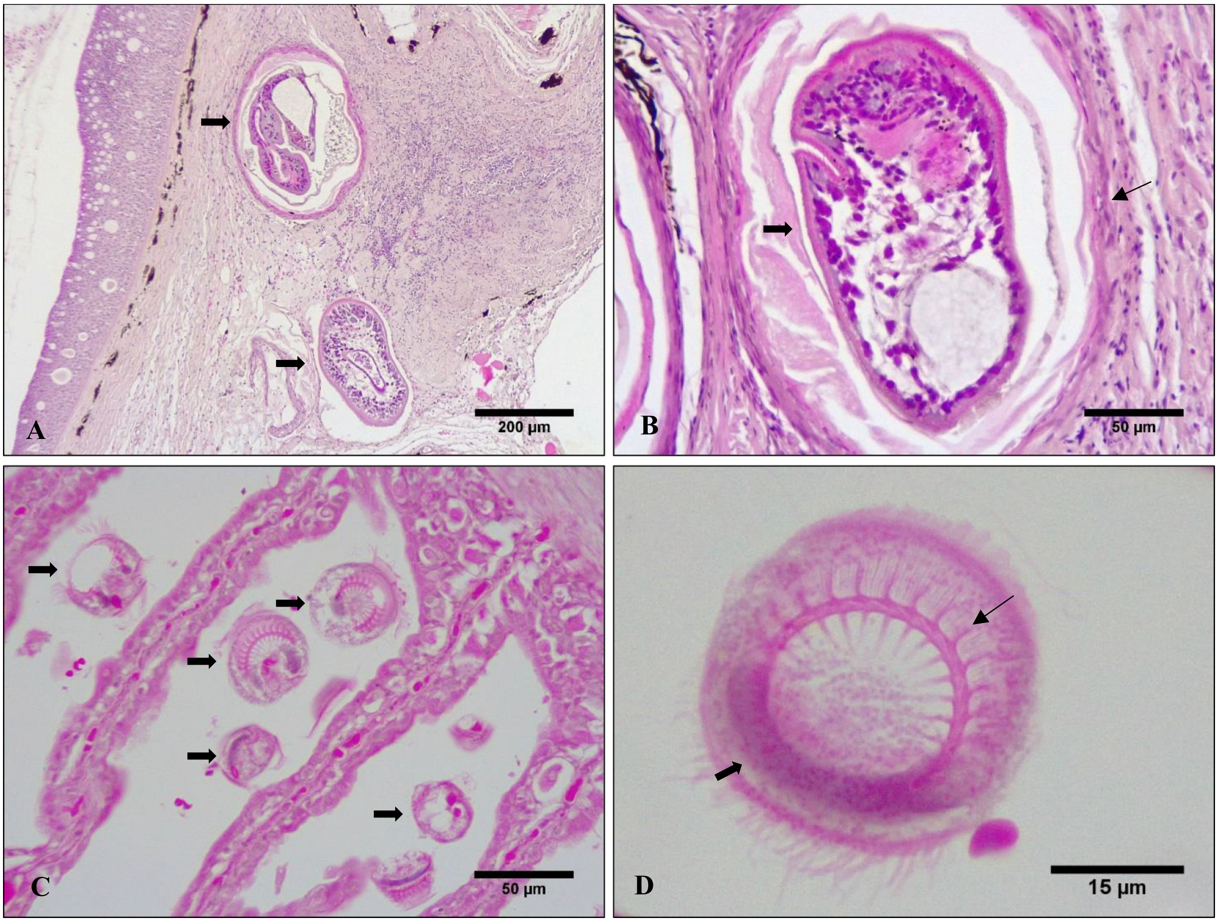

Fig. 3. Parasites in shorthorn sculpins from Maarmorilik, West Greenland. A. Digeneans (arrows) located in dermis. B. A digenean (thick arrow) isolated by host response (fibrosis, thin arrow). C. Trichodinids (arrows) between gill lamellae of shorthorn sculpins. D. A trichodinid with a part of typical horseshoe shape macronucleus (thick arrow) and adhesive disc denticles (thin arrow).

stations. There was no significant effect of station on mean barrier status of the skin (P = 0.432, Fig. 5C).

3.4.2. Skin mucosa and hepatic metals

The skin mucosal epithelium acts as the shielding barrier and may or may not be involved in excretion of heavy metals. In males, the size of skin mucous cells was positively correlated with hepatic level of Cr (R = 0.833, n = 6, P = 0.04).

3.4.3. Skin mucosa and parasite loads

Skin mucosa responded to parasites. In both sexes, a weak positive correlation between skin barrier status and the density of digenea on

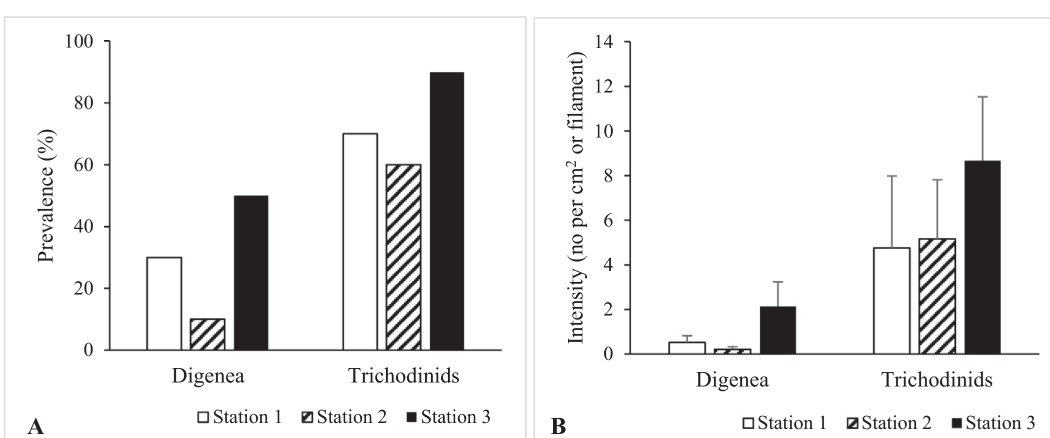

Fig. 4. Prevalence and intensity of infection with digeneans (skin) and with trichodinids (gills) of shorthorn sculpins from stations along a distance gradient near the former Pb—Zn mine at Maarmorilik, West-Greenland (from histological examination). A. Prevalence. B. Intensity (mean + SE).

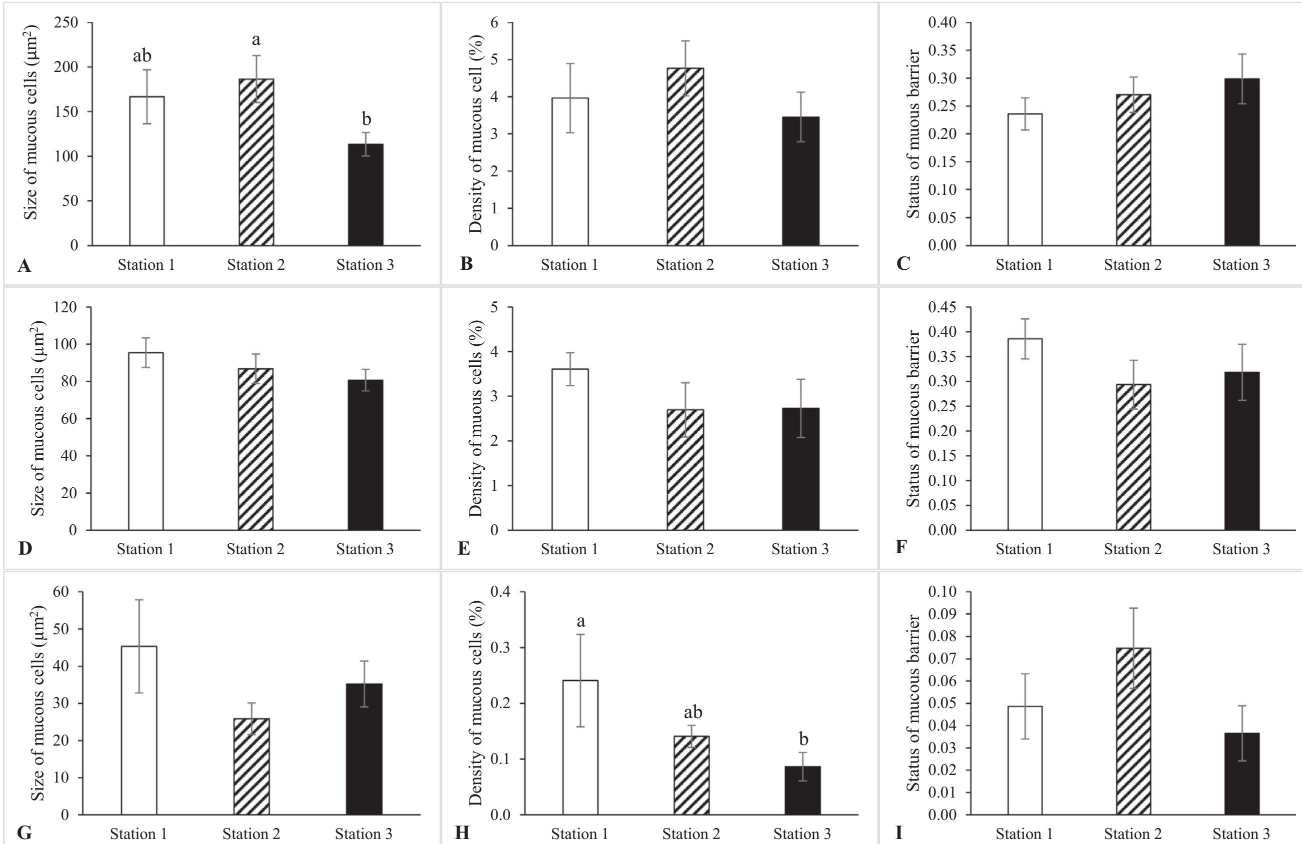

Fig. 5. Maps of mucous cells on skin, gills of shorthorn sculpin (n = 30) at the 3 stations. A. Size of skin mucous cells. B. Density of skin mucous cells. C. Barrier status of skin mucous cells. D. Size of filamental mucous cells. E. Density of filamental mucous cell. F. Barrier status of filamental mucous cells. G. Size of lamellar mucous cells. H. Density of lamellar mucous cell. I. Barrier status of lamellar mucous cells. Data are presented as Mean ± SE. Means with different letters are significantly different from each other (P < 0.05).

the skin was evident (R = 0.56, n = 15, P = 0.029). For males, a strong positive correlation between barrier status and digenea infestation was seen (R = 0.91, n = 6, P = 0.012) as was the correlation between density of digenea and skin mucous cell density (R = 0.83, n = 6, P = 0.042). In females, no significant correlations were found (n = 9, P > 0.05).

3.4.4. Gills

On average, mucous cells in the gill filaments were 87.63 ± 5.75 μm^2 in size and at a density of 3.01 ± 0.65% of the volume of the epithelium layer giving a barrier status of 0.33 ± 0.06. There was no significant difference in size (P = 0.33), density (P = 0.40) or barrier status (P = 0.36) of mucous cells on the filaments of shorthorn sculpin between the three stations (Fig. 5D–F).

Notably, the lamellar mucous cells showed a significant difference (P = 0.047; Fig. 5H) in volumetric density between fish from station 1 (0.24% of epithelium layer) and those from station 3 (0.09%). Neither the size nor the barrier status of gill lamellar mucous cells varied significantly along the contamination gradient (P = 0.25 and 0.18, respectively, Fig. 5G, I).

3.4.5. Gill mucosa and hepatic metals

The filament showed a significant positive correlation between the size of mucous cells and the concentration of Pb in liver of both sexes (R = 0.69, df = 15, P = 0.004) (Fig. 6). For males, gill filament mucous cell sizes and the liver Pb levels were significantly correlated (R = 0.92, df = 6, P = 0.009) whereas in females this correlation between filament cells and liver concentrations was absent (R = 0.65, df = 9, P = 0.06). Instead, the lamellar mucous cell sizes in females showed a significant positive correlation with hepatic Pb level (R = 0.70, df = 9, P = 0.037).

3.4.6. Gill mucosa and parasite load

Though a high level of parasite infections was observed on the gills of sampled fish, there was no correlation between any gill mucosal indices and parasite load (P > 0.05).

4. Discussion

4.1. Interaction between fish and metals

Almost three decades after the mine closure, sites near the Black Angel mine are still contaminated with heavy metals. This is shown by

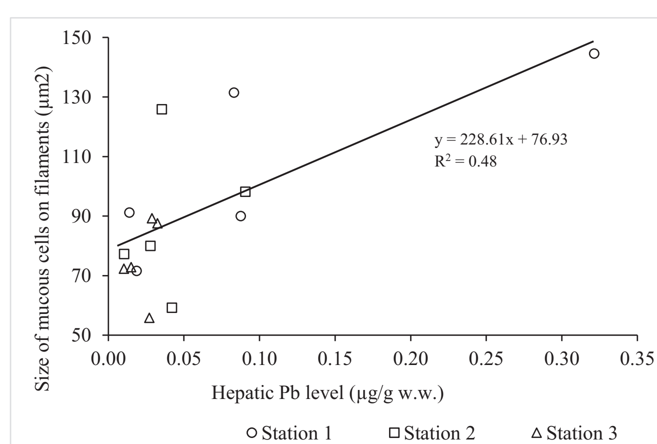

Fig. 6. Correlation between hepatic lead (Pb) level and size of filamental mucous cells.

high levels of Pb (22 fold greater than PELs) and Zn (6 time greater than PELs) in bottom sediment and as dissolved labile metal in seawater (although less pronounced than for sediment) close to the mine (station 1) compared to 12 km away (station 3). Bottom dwelling stationary shorthorn sculpin are affected by local contaminants not only via direct exposure with contaminated sediment but also through diet (sediment-feeding worms, crabs, and other benthic organisms) (Scott and Scott, 1988). Because Pb and Zn have distinct differences in biological requirement, uptake, metabolism, excretion and regulation (Wood et al., 2011a, 2011b), shorthorn sculpins would respond differently to high levels of Pb than to Zn in sediment.

Pb is a non-essential element which has no biological function in fish and is passively taken up via gills, skin and intestine that are all covered by mucous barrier (Mager, 2011). An increase in production of mucus was a common response after exposure to Pb (Bols et al., 2001; Mager, 2011). For example, when fish (*Carassius carassius* and *Leuciscus phoxinus*) were exposed to lethal concentrations of Pb (20–100 mg/L), a thin coat of mucus covered the body and a thick pad of mucus was found within operculum (Carpenter, 1927). The mucus excretion could cause respiratory stress, irregular ventilation and swimming. Exposure to a moderate level of Pb also increased mucus excretion but the health effects are less severe (Carpenter, 1927; Jones, 1938; Varanasi and Markey, 1978; Westfall, 1945). In the present study, the gill lamellae of sculpins at station 1 were distinct from those at the other stations by a higher mucous density. Because in fish gill lamellae are the area for respiration (Evans et al., 2005), the statistically significant higher density of mucous cells on lamellae of fish from station 1 could result in an increased respiratory diffusion distance and reduced area for contact between water containing oxygen and blood. By contrast, the gill filament cell populations displayed no difference to the environment as neither mucous cell areas, densities nor barrier status of gill filaments were significantly different between stations.

Furthermore, in our study, a significant positive correlation between level of Pb in liver and size of filamentous mucous cells was found. This means fish with high level of Pb accumulated in liver tended to have larger filamentous mucous cells. Since the excretion of Pb can occur in gills (Mager, 2011) and there is some evidence that coho salmon (*Oncorhynchus kisutch*) excrete Pb within mucus (Varanasi and Markey, 1978), the larger filamentous mucous cells in fish with high level of hepatic Pb can be an active way to reduce the somatic load of Pb in these fish. Mucosal immunoglobulins were implicated in gill transport mechanisms from the epithelium to the blood capillaries of the gills in rainbow trout (*Oncorhynchus mykiss*) and the trout polymeric immunoglobulin receptor gene (tpIgR) was primarily expressed in the gill filament (Xu et al., 2016). Taken together the results imply a distinct function of the gill filament mucous cells in accumulation and transport of somatic toxins to the exterior, in addition to mucosal immunity.

4.2. Interaction between parasites and metals

Exposure to pollutants may either increase or decrease parasitism (Sures, 2008; Sures et al., 2017). *Trichodina* spp. did not proliferate in environment contaminated with Pb. A study conducted in tilapia (*Oreochromis niloticus*, $n = 330$) found that 65% of fish caught from control site (Pb: 0 ppm) were infected by trichodinids whereas only about 48% of the fish from contaminated stations (Pb: 0.5 ppm) were infested (El-Seify et al., 2011). Similarly, lower levels of trichodinids infection were observed in catfish (*Clarias gariepinus*, $n = 140$) from sites with higher level of Pb in water (Pb 0.5 ppm) (El-Seify et al., 2011). A decrease in intensity and vitality of *Trichodina* sp. was reported in tilapia after 30 days of exposure to lead acetate (10.1–20.2 ppm) (El-Bouhy et al., 2016). Hence, in our study, environmental Pb levels may play a role in reducing trichodinid parasitism at the most contaminated site. At the same time, it cannot ignore that this is a field study so a number of other factors (rather than just Pb) such as salinity, temperature, nutrition and oxygen deficiency can possibly affect trichodinid infection.

Similar to trichodinid infection, both prevalence and intensity of digenean infection in sculpin from station 3 (the least polluted station) were higher those in station 1 (the most polluted station). These results agreed with literature on interactions between pollution and parasites (Khan and Thulin, 1991; Mackenzie, 1999; Sures, 2008; Sures et al., 2017). Heteroxenous parasites with complex life cycles such as digeneans might be more vulnerable to an exposure to contaminants because of their dependency on intermediate and final hosts and their free-living life stages which may be susceptible to a changing environment (Khan and Thulin, 1991; Mackenzie, 1999; Sures, 2008; Sures et al., 2017). As a general rule, infection levels with endoparasitic helminths such as digeneans, cestodes and acanthocephalans decrease when the hosts are exposed to most types of pollution including heavy metals (Filipović Marijić et al., 2013; Kennedy, 1985; Mackenzie, 1999; Sures, 2008; Sures et al., 2017). Lower level of infection in fish from station 2 may be related to highest level of Zn in water from this station (Table 1). Zinc is an essential elements and necessary for active proliferation and differentiation of epidermal cells as well as stimulating re-epithelialization, modulating inflammation, stimulating wound healing and removal of cells affected by necrosis (El-Adl et al., 2018; Fosmire, 1990; Jensen et al., 2015). The improvement in these functions would benefit fish in interaction with parasite infection.

4.3. Interaction between host and parasite

4.3.1. Skin

In this study, metacercariae were found in the underling muscle and dermal layer of skin. Digeneans (*Derogenes varicus*, *Hemiurus levinseni*, *Podocotyle atomon*, *Steringophorus furciger*, *Cryptocotyle lingua*, *Phyllodistomum undulans*, *Crepidostomum cooperi*, *Crepidostomum farionis*, *Diplostomum spathaceum*, *Diplostomum* sp., *Neascus* sp. and *Tetracotyle* sp.) were reported in a number of sculpin species including slimy sculpin (*Cottus cognatus*), shorthorn sculpin, longhorn sculpin (*M. octodecemspinosus*) and mottled sculpin (*Cottus bairdi*) (Barker et al., 1994; Khan, 2011, 2012; Muzzall and Bowen, 2002; Muzzall and Koslosky, 2010; Muzzall and Sweet, 1986). In general, the life cycle of a digenean include: (1) egg, (2) miracidium, (3) sporocyst, (4) redia, (5) cercaria, (6) metacercaria and (7) adult. Fish can be both intermediate and definitive hosts and the infection with digenea into fish occurs at cercaria stage (free swimming stage). Cercariae can either directly penetrate into fish via skin, fin, gills, eye or being ingested into fish's gut then start the next stage (Bullard and Overstreet, 2008). In shorthorn sculpin, adult digeneans were found in digestive tract (*Derogenes varicus*, *Hemiurus levinseni*, *Podocotyle atomon* and *Steringophorus furciger*) and metacercariae (*Cryptocotyle lingua*) were found in the gills (Khan, 2004, 2011). Metacercariae of *C. lingua* were numerous in skin and gills of winter flounder (*Pleuronectes americanus*) (Khan, 2004) and cercariae of *C. lingua* could penetrate through the skin of the cunner (*Tautogolabrus adspersus*) under experimental conditions (Stunkard, 1930). Penetrating cercariae can cause local irritation of the host, this has been shown for the *C. lingua* in the cunner (*Tautogolabrus adspersus*), *Procerovum cheni* in Japanese eels (*Anguilla japonica*) and *Transversotrema patialense* in zebra danio (*Danio rerio*) (Bullard and Overstreet, 2008; Stunkard, 1930). In the present study, the presence of metacercariae in dermis and the underlying muscle of shorthorn sculpin suggest that cercariae may directly infect into fish via skin. To directly penetrate into the fish via skin, cercariae need to pass mucous barriers. In this study, shorthorn sculpin from station 3 which had smaller size of mucous cells on the skin had a higher parasitic infection level. This result revealed an interesting dynamic interaction between host and parasite.

The smaller size of mucous cells in station 3 could be due to a first and active response of fish to get rid of the attack of parasites. It is hypothesised that fish would respond to the environmental challenges (such as parasite) firstly in cell size then followed by a cell density (Torrecillas et al., 2015). A smaller size of mucous cells could be an active response of fish to fight off parasites because these smaller mucous cells can fill quicker and move quicker to surface area to wash off pathogens including parasites. Mucus clearly plays a role in limiting parasite loads, since a higher numeric density of mucous cells is significantly correlated with lower monogeneans in rainbow trout (Buchmann and Bresciani, 1998). An important distinction is made between numeric and volumetric density: while the former can increase if the cells become more numerous, the volumetric density will vary with the number and size of the cells in a reference volume. Thus, in numeric density there can be a "reduction" with one large cell being "less dense" than 5 very small ones, whereas volumetric density will reflect the filling of the epithelium by cells of a known size. The present study reported skin mucous cell volumetric density which was relatively constant at around 4% despite significant differences in size and therefore in number.

Both monogeans and crustacean ectoparasites can reduce the numeric density of mucous cells in the skin potentially reducing the inhibitory effect of inflammatory components and complement factors in mucous secretion on parasite load (Jones, 2001). If the challenges overwhelm resources, the numeric density of mucous cells in skin would decrease, indicating an exhausted status (Ledy et al., 2003). For example: chronic exposure to sub-lethal stress induces fish to release abundant mucous onto the surface to get rid of stressors resulting in fewer mucous cells in epithelial layer (McKim and Lien, 2001). However, in our study, the skin mucous cell density was stable between the three stations, suggesting a balance between challenge and response. This further suggests that barrier status of fish from station 3 was not impaired by the combination of metals and digenea infestation. However, the correlation existing between skin barrier status, involving mucous cell size and volumetric density, and digenea density suggests that the shorthorn sculpin skin "shield" continuously reacted and responded to the digenea infection to maintain a balance between host and pathogens.

4.3.2. Gills

In this study, only fish from station 3 had a high prevalence of ectozoic trichodinid infection (90%) at a moderate density (10 individual/cm of filament) while the mucous cells on gill filaments and lamellae showed no correlation to infestation levels. No significant association between trichodinid infection and any gill mucosal indices was evident. Trichodinids are commonly reported in sculpin (Dang et al., 2017; Khan et al., 1994; Khan and Thulin, 1991; Muzzall and Bowen, 2002; Muzzall and Sweet, 1986; Nørregaard et al., 2018) and other aquatic animals (Lom and Dyková, 1992). Basically, this parasite eats waterborne or detrital particles, microbiota on surface of the host or ruptured cells of the host including mucous cells (Lom and Dyková, 1992). The presence of ectozoic trichodinids at a high prevalence and low density may not be detrimental to the health of the sculpin.

5. Conclusions

The sculpin's mucosal epithelia of skin and gills reacted to environmental pollution and parasites. Skin mucous cells were significantly smallest in the sculpin caught at station 3, where Pb level was lowest and skin parasite load was highest. Importantly, there was a positive correlation between size of gill filament mucous cells and Pb concentrations in liver. The density of gill lamellar mucous cells followed a Pb gradient and was significantly greatest in the sculpin caught at the most polluted station and lowest at the least polluted station. To the best of our knowledge this is the first time an ecotoxicological study has been able to quantify in a statistically robust manner volumetric density of mucous cells in the skin and gills in a bioindicator fish species. These mucosal epithelial changes can be used to determine fish adaptations and differential responses to environmental challenges in field studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.04.412.

Acknowledgments

This project was funded by financial support from The Ministry of Environment and Food of Denmark, The Environmental Agency for Mineral Resource Activities (project 771020), Government of Greenland, and the Arctic Research Centre, Aarhus University, which are all greatly acknowledged. The authors also wish to thank Adrian Hehl for assistance in the field, as well as the laboratory technicians Anna Marie Plejdrup and Thomas Hansen for assistance with the Q-ICP-MS analysis. In addition, the mining company Angel mining PLC is acknowledged for providing the logistical facilities on site during the 2017 summer sampling campaign.

The authors sincerely thank the Australian Society for Parasitology for financial support (via a JD Smyth Postgraduate Student Travel Award 2017) for the Norway trip to analyse samples. Dr. Mach Diep, Dr. Nguyen Thi Hai Thanh, Dr. Le Hoang Thi My Dung, and Dr. Mei Ooi are acknowledged for their assistance and encouragement. Dr. Grigory Merkin and Dr. Mearge Okubamichael of Quantidoc AS, Bergen, Norway, are gratefully acknowledged for their assistance, patience and insight during the analyses of mucous cells.

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