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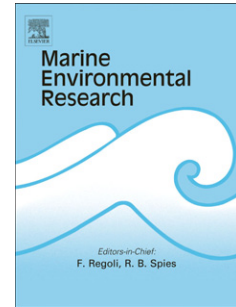
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1 **Seasonal variation of histopathological and histochemical markers of**
2 **PAH exposure in blue mussel (*Mytilus edulis* L.)**

3
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6

7 **Abstract**

8 The aim of this work was to study seasonal variation of histopathological and
9 histochemical markers in blue mussels (*Mytilus edulis* L.) exposed to pyrogenic PAH
10 contaminants. Mussels were collected in January 2007, June 2006, September 2006
11 and October 2007 from a sampling site in the vicinity of the discharge from an
12 aluminium smelter and from a clean reference site. Histopathological analysis was
13 carried out on the digestive gland (DG) and the gonads. Lipofuscin and neutral lipids
14 were analysed in the DG. Clear responses in lipofuscin and neutral lipids were detected
15 in the DG of mussels collected from the polluted site at some sampling times.
16 Moreover, these mussels presented atrophy in digestive tubules and haemocytic
17 aggregates in the gonad and DG. However, in all parameters studied, the magnitude of
18 the response showed clear seasonal variation.

19
20 Key words: Histopathology, neutral lipid, lipofuscin, PAH, *Mytilus edulis*, seasonal
21 variation.

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25

26 **1. Introduction**

27 Mussels are often used to evaluate the effect of contaminants in biomonitoring programs
28 due to their ability to survive under polluted conditions and to accumulate both organic
29 and metal pollutants (Lowe and Pipe, 1994; Moore, 1988; Viarengo, 1985). Biomarkers
30 are often used to reflect the changes in cells and tissues caused by environmental
31 contamination. Responses in such markers indicate that the organisms have been
32 exposed to a pollutant (exposure biomarkers) and/or the magnitude of the organisms'
33 response to the pollutant (biomarkers of effects). Histopathology is a biomarker of
34 effects and may be used to identify pathological lesions in tissues induced by
35 environmental contamination and disease. These changes reflect disturbances at the
36 molecular level (Moore and Simpson, 1992) and can aid in the understanding of the
37 overall health of the animals. Histopathology is often the easiest method for assessing
38 both short- and long-term toxic effects in the field (Hinton and Laurèn, 1990) by
39 providing an overall assessment of the general health status of mussels.

40 Lipofuscin (LF) and neutral lipid (NL) are biomarkers of effects often used in
41 biomonitoring programs. NL is also considered as an exposure biomarker against
42 organic chemical compounds (Marigomez and Baybay-Villacorta, 2003). These
43 parameters are analysed in the digestive gland (DG) which can be affected by many
44 pollutants (Axiak et al., 1988; Lowe, 1981; Lowe, 1988; Marigómez et al., 1986 ;
45 Syasina et al., 1997; Tripp, 1984). The DG is the main site for metabolic activity; it is
46 involved in immune defence, detoxification and in homeostatic regulation (Marigomez
47 et al., 2002; Moore and Allen, 2002). The DG consists of many blind-ending tubules
48 which are lined by a digestive epithelium, made up of digestive and basophilic cells.

49 The lysosomes in the digestive cells have been identified as a target site for many
50 contaminants (Cajaraville et al., 1995; Moore, 1990).

51 LF is the end-product in the peroxidation process and is accumulated as insoluble
52 granules in the lysosomes. These granules consist of oxidatively modified proteins, lipid
53 degradation products, carbohydrates and metals (Terman and Brunk, 2004; Viarengo et
54 al., 2007), and the amount of accumulated granules indicates the oxidative stress levels
55 (Viarengo and Nott, 1993). This biomarker is influenced by seasonal temperature
56 changes and physiological processes such as the reproductive cycle (Bocchetti and
57 Regoli, 2006; Petrovic et al., 2004; Regoli, 1992). In addition, NL is a useful indicator
58 for the alterations of cell physiology (Koehler, 2004; Viarengo et al., 2007). The
59 accumulation of NL in lysosomes has been found to be a useful indicator of pollutant
60 exposed organisms (Moore, 1988).

61

62 In a previous paper, (Aarab et al., 2008) have reported that both LF and
63 histopathological examination of the DG, showed a strong response in mussels collected
64 from a pyrogenic PAHs polluted site. This present study is a following up of this work
65 and the aim was to investigate the seasonal variation of histopathological and
66 histochemical changes in mussels exposed to PAH contamination.

67 **2. Materials and Methods**

68 **2.1 Sampling**

69 Blue mussels, *Mytilus edulis*, were collected at two sites in the Southern part of Norway
70 between June 2006 and October 2007. The water salinity in both sites ranges between
71 35.1‰ and 35.3‰. Site 1 (Førlandsfjorden) is a clean reference site and Site 2

72 (Høgevarde) is slightly north of the discharge from an old aluminium smelter in
73 Karlsund. The site had a production of about 200,000 tons of aluminium per year, and
74 the discharge of PAH to the Karlsund strait was approximately 450 kg annually
75 (Figure 4) (Beyer et al., 1998; Aarab et al., 2008). Previous results from mussels
76 collected in March and April 2006 are already published (Aarab et al., 2008). In this
77 follow-up paper we present results from mussels collected on four periods intended to
78 encompass seasonal variation. The mussels were sampled by scraping at 1 m depth the
79 following dates; 14. June 2006, 01. September 2006, 08. October 2007 and 16. January
80 2007. The number of mussels collected from each site varied between 15 to 30
81 specimens ($6.0 \text{ cm} \pm 0.5 \text{ cm}$).

82 **2.2 Histopathology**

83 During dissection, mussels were examined for the presence of parasites, pearls and other
84 tissue abnormalities. The gonads and DG were dissected and placed into individual
85 histocassettes, fixed in Baker's calcium solution (4% formaldehyde, 1% CaCl_2 , 2, 5%
86 NaCl) and dehydrated in alcohols. The tissues were cleared in methyl benzoate
87 ($\text{C}_6\text{H}_5\text{CO}_2\text{CH}_3$), rinsed in benzene (C_6H_6) and embedded in paraffin. Histological
88 sections ($5 \mu\text{m}$ thickness) were cut using a microtome HM 355s (Microm, Bergman),
89 mounted on slides, dried at 37°C for 24 hours and stained with hematoxylin and eosin.

90 The tissues were examined for health parameters related to reproductive and
91 physiological conditions, inflammatory and non-specific pathologies and those
92 associated with pathogen and parasite infections. The DG tubule atrophy was recorded
93 using a scoring index ranging from 0 to 3 (Brooks et al., 2009); 0: tubules nearly
94 occluded, slight atrophy in few tubules, 1: slight atrophy to one half normal tubule
95 thickness, 2: significant atrophy to one half tubule thickness and 3: extremely thin, most
96 tubules affected. The reproductive status was determined according to (Seed, 1976) as

97 resting gonad (score 0), developing gonad (score 1-4), ripe gonad (score 5) or spawning
98 gonad (score 1-4). The presence of parasites and non-specific inflammation were scored
99 as absent (0) or present (1). 15 to 30 individuals were examined from each site, and 4
100 sections per individual were analyzed to score parasites, inflammation the tubule
101 atrophy. Micrographs were captured using an AxioCam MRc5 (Zeiss) digital camera
102 mounted on a *Zeiss Axioplan 2* light microscope (Göttingen, Germany).

103 **2.3. Histochemistry**

104 Small pieces of freshly excised DG tissues were placed on metal cryostat chucks. Each
105 chuck was placed for less than 1 min in a pre-cooled (-70 °C) bath of n-hexane. The
106 metal chucks were then sealed by double-wrapping in parafilm and aluminum foil and
107 stored at -40 °C. Cryostat sections (8 µm thickness) were cut in a cryostat Microm HM
108 560 (Bergman) and transferred to slides (20 °C), and then stored at -40 °C until use.

109 **2.3.1 Lipofuscin (LF)**

110 The accumulation of LF was determined using the Schmorl's reaction (Lowe, 1988).
111 The cryostat sections were fixed for 15 min in Baker's calcium solution at 4 °C, rinsed
112 in distilled water and stained for 5 min in an aqueous solution of 1% ferric chloride, 1%
113 potassium ferrocyanide and distilled water [(FeCl₃)/(K₄[Fe(CN)₆])/H₂O (7.5:1:1.5)].
114 The sections were rinsed in 1% acetic acid (CH₃COOH) for 1 min and washed in
115 distilled water before mounting in an aqueous mounting medium.

116 **2.3.2 Neutral lipid (NL)**

117 The determination of NL in digestive cells was carried out by the Oil red O method
118 (Bancroft, 1967). The cryostat sections were fixed for 15 min in Baker's calcium
119 solution at 4 °C, rinsed in distilled water and transferred to a 60% solution of triethyl
120 phosphate ((C₂H₅)₃PO₄) for 5 min. The sections were then stained with Oil Red O for 15

121 min, rinsed in 60% triethyl phosphate, washed in distilled water and mounted in an
122 aqueous mounting medium.

123 **2.3.3 Optical density assessment**

124 The average optical density of LF and NL were calculated from 2 micrographs per
125 individual (400x magnification) using a *Zeiss Axioplan 2* light microscope (Carl Zeiss,
126 Göttingen, Germany) and the image analysis program AxioVision 4. Fifteen to thirty
127 individuals were examined from each site.

128 **2.4 Statistical analysis**

129 JMP^R statistical computer software version 5.1 (SAS Institute, Cary, NC, USA) was
130 used to compare histopathological health parameters and the accumulation of LF and
131 NL between reference and polluted station. Analysis of variance (one way ANOVA)
132 and Control Dunnett's test were used to estimate significance. A significance level of
133 $P < 0.05$ was applied in all statistical tests.

134

135 **3. Results**

136 **3.1 Histopathology**

137 **3.1.1 Reproductive stage**

138 The median score of gonadal stage of mussels from site 1 was highest in January 2007
139 (4), followed by a decrease during June 2006 (3) and September 2006 (2), before
140 increasing again in October 2007 (3). The lowest maturation levels were recorded in
141 September 2006. The median gonadal stage of mussels from site 2 was highest in

142 January 2007 and June 2006 (3), decreasing in September 2006 (1, 5) and October 2007
143 (1).

144 3.1.2 Non-specific inflammation

145 Aggregations of haemocytes were found in gonadal follicles and digestive tubules in
146 mussels from both sites during this study (Figure 1). The observations were more
147 frequent in mussels collected from the polluted site (site 2) (55-95%) during all
148 sampling periods, compared to mussels collected from reference site 1 (5- 50%).

149 INSERT FIGURE 1

150

151 3.1.3 Digestive gland pathology

152 Digestive tubule atrophy was observed in mussels from both sampling sites (Figure 3).
153 Mussels collected from site 2 were more severely affected than mussels collected from
154 site 1 in all sampling periods (Figure 2).

155 INSERT FIGURE 2

156 3.1.4 Parasite infections

157 Three parasites were observed in mussels during the study: *Marteilia* sp., *Steinhausia*
158 *mytilovum* and digenean metacercaria (Figure 2). The number of mussels infected by
159 both *Marteilia* and digenean metacercaria at site 1 varied throughout the year, with the
160 lowest number of infected mussels in September 2006 (0-4%). Mussels collected from
161 site 2 were highly infected in all sampling months (85-100%). *Steinhausia mytilovum*
162 was only observed in the oocytes of 3 individuals from site 1 in June 2006.

163

164 INSERT FIGURE 2

165

166 3.2 Histochemistry

167 The accumulation of LF in mussels from both sites increased from January 2007 until
168 September 2006 and decreased in October. The accumulation of NL in mussels from
169 site 1 was low in January 2007, increasing in June 2006 and then decreasing in
170 September 2006 before reaching a maximum in October. The accumulation of NL in
171 mussels from site 2 was low in January 2007 and stable during September 2006 and
172 October 2007 (Table 1).

173

174 INSERT TABLE 1

175 4. Discussion

176 4.1 Histopathology

177 The high frequency of haemocytic aggregates observed inside the gonadal follicles and
178 DG tubules in mussels from the pyrogenic PAHs contaminated site might be due to the
179 presence of contaminants. This confirms results in an earlier study on mussels from the
180 same site (Aarab et al., 2008). Mussels exposed to a mixture of oil, PAHs and
181 alkylphenols also showed the same abnormalities in the gonads (Aarab et al., 2004).
182 Inflammatory reactions in the gonadal follicles have earlier been associated with
183 infections of *Steinhausia mytilovum* (Bignell et al., 2008). There were only three
184 *Steinhausia*-infected mussels in our study, so this may not explain the observed
185 inflammatory reactions. Haemocytic aggregates in the digestive tubules have been
186 associated with infections of *Marteilia* sp. and digenean metacercaria (Bignell et al.,

187 2008). In our study, mussels from both sites were infected with these parasites and the
188 prevalence does not seem to agree with the number of mussels with an inflammatory
189 reaction.

190 The seasonal fluctuations in digestive tubule atrophy observed in reference mussels
191 have earlier been reported (Bignell et al., 2008). However, these fluctuations were not
192 observed in mussels collected from site 2. In addition to that, the same mussels present a
193 higher score than reference mussels. These observations may be explained by the
194 presence of PAH compounds (Moore and Clarke, 1982; Aarab et al., 2008). Infections
195 of *Marteilia* sp. may cause atrophy to digestive tubules (Bignell et al., 2008), but this
196 does not seem to be the case in the present study.

197 **4.2 Histochemistry**

198 The seasonal variation of accumulated LF in mussels from both reference and PAH
199 contaminated sites is probably due to a reduced metabolism in October 2007 and
200 January 2007, caused by the effect of temperature and food availability (Bocchetti and
201 Regoli, 2006; Gorbi et al., 2008; Petrovic et al., 2004; Regoli, 1992). However, in June
202 2006 and September 2006 the accumulation of LF in mussels from the polluted site was
203 higher than reference mussels. This might indicate a stress response to the
204 environmental conditions at this site. These results are in agreement with previous
205 studies that have reported that accumulation of LF is a very sensitive biomarker of stress
206 to PAHs (Da Ros et al., 2007; Pipe and Moore, 1986; Aarab et al., 2008), to industrial
207 and domestic waste water (Domouhtsidou and Dimitriadis, 2001; Petrovic et al., 2004)
208 and to heavy metals contamination (Krishnakumar et al., 1990; Krishnakumar et al.,
209 1994; Regoli, 1992; Viarengo et al., 1987).

210 The seasonal variation of NL accumulation seen in mussels collected from site 1 might
211 be explained by the reproductive cycle of the mussels. During the spawning season the
212 individuals move the NL from the DG to the gonad before spawning (Abad et al., 1995;
213 Cancio et al., 1999; Regoli et al., 1992; Thompson et al., 1974). Mussels collected from
214 the PAHs contaminated site did not seem to follow the same seasonal trend as the
215 reference mussels did, and they show a lower accumulation of NL in September 2006
216 and October. This low accumulation of NL might be caused by the environmental stress
217 since there was no difference in the gonadal stage in June 2006. Similar results in PAHs
218 and copper exposed mussels have earlier been observed (Zorita et al., 2006). Under
219 environmental stress mussels use their reserve materials from the DG during the
220 detoxification process (Regoli et al., 1992; Thompson et al., 1974). In agreement with
221 (Cancio et al., 1999) the low accumulation of NL in January 2007 is probably due to
222 the low food availability during this season.

223 One key issue raised from this study is that digestive tubule atrophy and haemocytic
224 aggregates in the DG can be used as an effect parameter for pyrogenic PAHs. However,
225 due to seasonal variation comparison of results between sites should relate to the same
226 sampling time during the year. Presence of such lesions may lead to misinterpretation of
227 NL accumulation, due to anatomical alterations in the cells.

228 **Conclusion**

229 In conclusion, the present study has demonstrated that lipofuscin and neutral lipids,
230 together with histopathological analysis of gonads and digestive gland are useful
231 parameters of stress caused by the pyrogenic PAHs. However, the seasonal variation,
232 combined with the health status and the reproductive stage of the organisms, needs to be
233 considered during interpretation. If these factors are taken into account, the

234 histopathology and histochemistry are suitable tools to be used in PAHs environmental
235 monitoring programs. However, based on our results, we would recommend that
236 sampling for monitoring purposes should be carried out during the same months each
237 year, and that comparison between years should be based on results generated in the
238 same season (Garmendia et al., 2010). For more comprehensive programmes the
239 sampling schedule should reflect seasonal variation in the selection of sampling months.

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ACCEPTED MANUSCRIPT

Research Highlights

- The present study has demonstrated that lipofuscin and neutral lipids, together with histopathological analysis of gonads and digestive gland are useful biomarkers of stress caused by the pyrogenic PAHs.
- For more comprehensive biomonitoring programmes the sampling schedule should reflect seasonal variation in the selection of sampling months (reproduction cycle).
- The seasonal variation, combined with the health status and the reproductive stage of the organisms, needs to be considered during interpretation.
- The histopathology and histochemistry are suitable tools to be used in PAHs environmental monitoring programs.

Table1. Optical density measurements recorded for lipofuscin and neutral lipids in mussels collected from reference site (site 1) and PAHs contaminated site (site 2). * $p=2.10^{-4}$, ** $p=2.10^{-8}$.

Figure1. Prevalence of non-specific inflammation, HA: Hemocytic aggregates in gonads and digestive gland (DG) of mussels collected from reference site (site 1) and PAHs contaminated site (site 2) during all sampling dates.

Figure2. Histological sections through the digestive gland and gonad of *Mytilus edulis* (a-d). (a) Digestive gland with a digenean metacercaria (arrow head) and digestive tubules (arrows) in a mussel collected from site 1. (b) Digestive gland with atrophic tubules (arrows) in mussel collected from site 2. (c) Hemocytic aggregates (arrows) within the testicular follicle in mussel from site 1. (d) Melanised hemocytic aggregate (arrow) within the testicular follicle and hemocytic aggregate in connective tissue (arrow head) in mussel from site 2. Hematoxylin and eosin (H&E) staining. Section: 5 μm thickness. Scale bare is given in lower right corner of the panel.

Figure3. Average score of digestive tubule atrophy in mussels collected from the reference site (site 1) and PAHs contaminated site (site 2) during all sampling dates. * $p=1, 3.10^{-2}$, ** $p=8.10^{-7}$.

Figure4. US EPA PAHs (in mg/kg wet weight given as average of six pools of 10 individuals from each locality) and st dev. The exposed locality Høgevarde (site 2) is showing a pyrogenic PAH profile whereas levels in the reference locality Førlandsfjorden (site 1) represents natural background levels (Aarab et al., 2008).

		Sampling months			
		January	June	September	October
LF (10^6)	Site 1	4.0	7.8	24.6	14.1
	Site 2	2.6	** 27.4	31.2	12.8
NL (10^7)	Site 1	1.2	8.1	6.6	10.5
	Site 2	2.2	* 4.8	4.7	** 5.1

Table1.

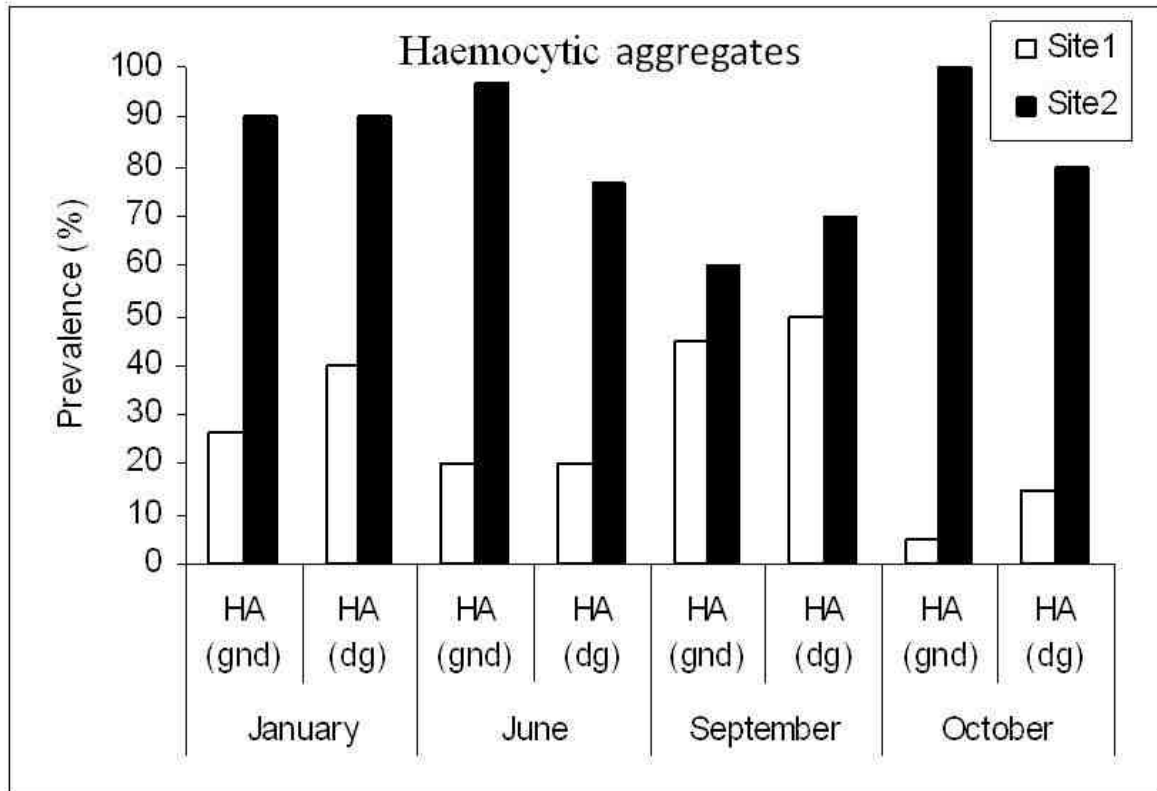


Figure 1.

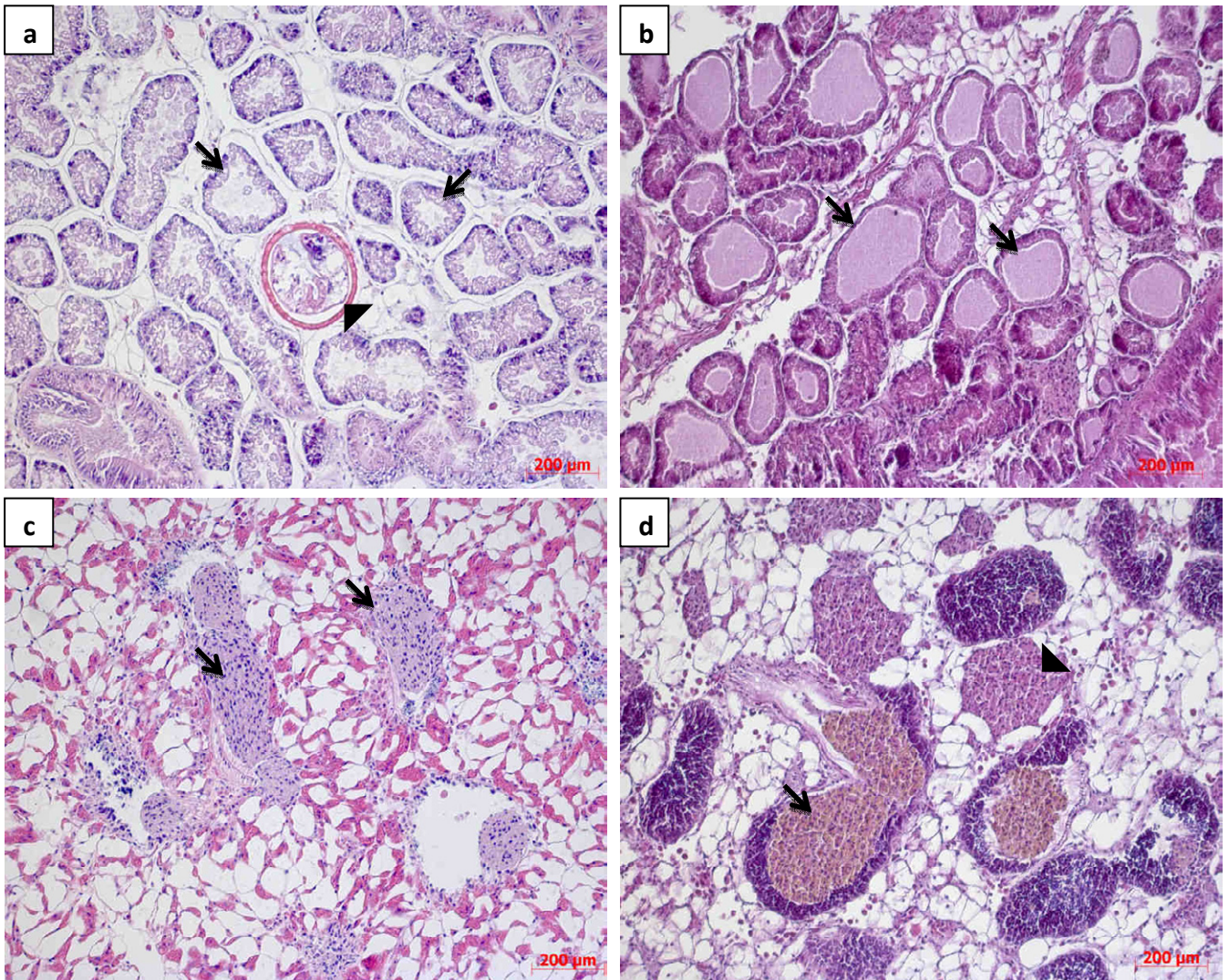


Figure 2

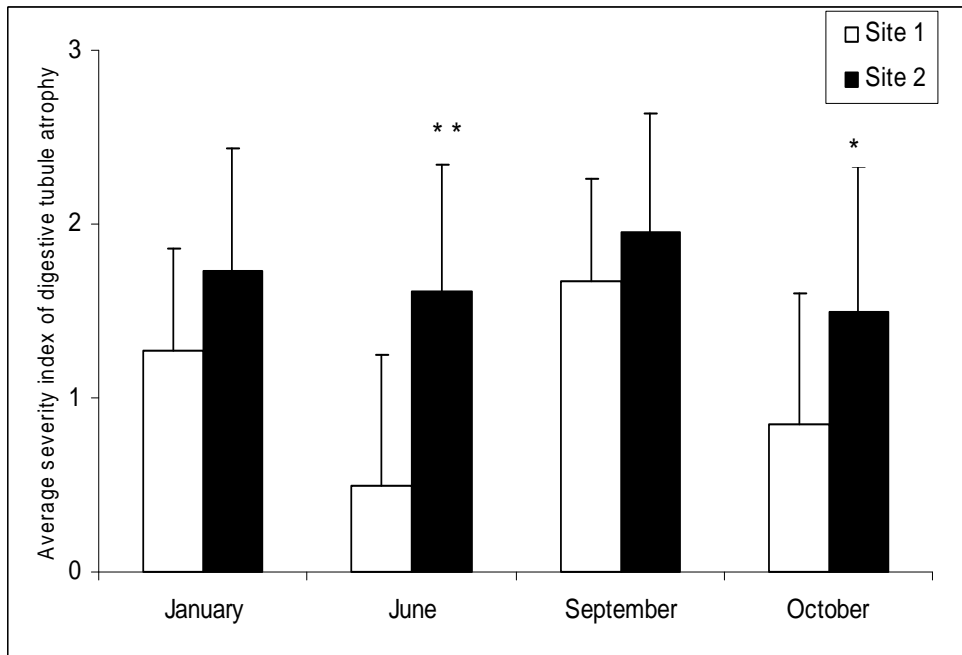


Figure3.

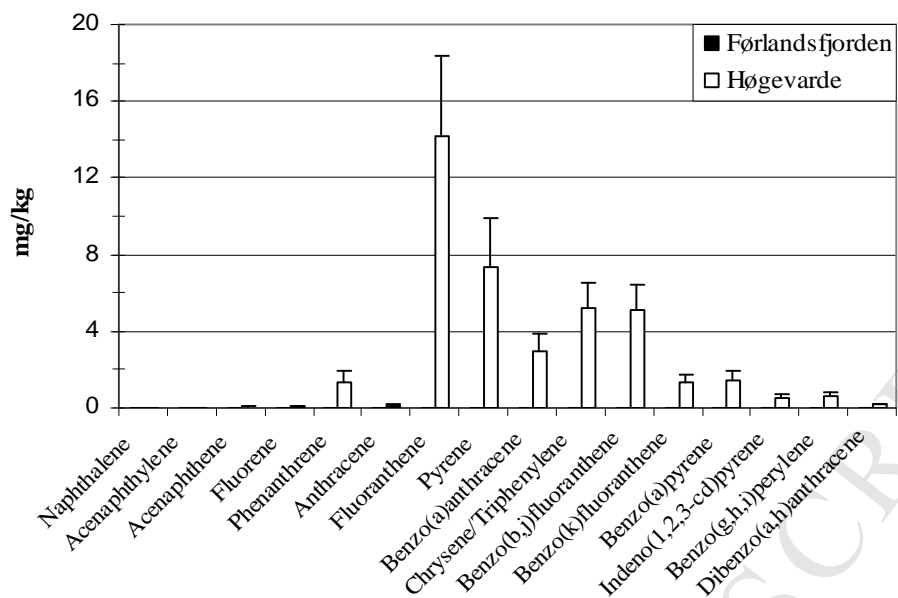


Figure 4. US EPA PAHs (in mg/kg wet weight given as average of six pools of 10 individuals from each locality) and st dev. The exposed locality Høgevarde is showing a pyrogenic PAH profile whereas levels in the reference locality Førlandsfjorden represents natural background levels. (Aarab *et al.*, 2008)