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

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**Abstract**

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

Key words: Histopathology, neutral lipid, lipofuscin, PAH, *Mytilus edulis*, seasonal 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.

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

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

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

## 2. Materials and Methods

### 2.1 Sampling

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

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

## 2.2 Histopathology

During dissection, mussels were examined for the presence of parasites, pearls and other tissue abnormalities. The gonads and DG were dissected and placed into individual histocassettes, fixed in Baker's calcium solution (4% formaldehyde, 1%  $\text{CaCl}_2$ , 2, 5%  $\text{NaCl}$ ) and dehydrated in alcohols. The tissues were cleared in methyl benzoate ( $\text{C}_6\text{H}_5\text{CO}_2\text{CH}_3$ ), rinsed in benzene ( $\text{C}_6\text{H}_6$ ) and embedded in paraffin. Histological sections ( $5 \mu\text{m}$  thickness) were cut using a microtome HM 355s (Microm, Bergman), mounted on slides, dried at  $37^\circ\text{C}$  for 24 hours and stained with hematoxylin and eosin. The tissues were examined for health parameters related to reproductive and physiological conditions, inflammatory and non-specific pathologies and those associated with pathogen and parasite infections. The DG tubule atrophy was recorded using a scoring index ranging from 0 to 3 (Brooks et al., 2009); 0: tubules nearly occluded, slight atrophy in few tubules, 1: slight atrophy to one half normal tubule thickness, 2: significant atrophy to one half tubule thickness and 3: extremely thin, most tubules affected. The reproductive status was determined according to (Seed, 1976) as

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

### 2.3. Histochemistry

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

#### 2.3.1 Lipofuscin (LF)

The accumulation of LF was determined using the Schmorl's reaction (Lowe, 1988). The cryostat sections were fixed for 15 min in Baker's calcium solution at 4 °C, rinsed in distilled water and stained for 5 min in an aqueous solution of 1% ferric chloride, 1% potassium ferrocyanide and distilled water [(FeCl<sub>3</sub>)/(K<sub>4</sub>[Fe(CN)<sub>6</sub>])/H<sub>2</sub>O (7.5:1:1.5)]. The sections were rinsed in 1% acetic acid (CH<sub>3</sub>COOH) for 1 min and washed in distilled water before mounting in an aqueous mounting medium.

#### 2.3.2 Neutral lipid (NL)

The determination of NL in digestive cells was carried out by the Oil red O method (Bancroft, 1967). The cryostat sections were fixed for 15 min in Baker's calcium solution at 4 °C, rinsed in distilled water and transferred to a 60% solution of triethyl phosphate ((C<sub>2</sub>H<sub>5</sub>)<sub>3</sub>PO<sub>4</sub>) for 5 min. The sections were then stained with Oil Red O for 15

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

### 2.3.3 Optical density assessment

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

## 2.4 Statistical analysis

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

## 3. Results

### 3.1 Histopathology

#### 3.1.1 Reproductive stage

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



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

### 3.1.2 Non-specific inflammation

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

INSERT FIGURE 1

### 3.1.3 Digestive gland pathology

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

INSERT FIGURE 2

### 3.1.4 Parasite infections

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

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.,

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

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

#### 4.2 Histochemistry

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

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

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

## Conclusion

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

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

## 5. Acknowledgement

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## 6. References

- Aarab, N., Minier, C., Lemaire, S., Unruh, E., Hansen, P.D., Larsen, B.K., Andersen, O.K., Narbonne, J.F., 2004. Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. *Marine Environmental Research* 58, 437-441.
- Aarab, N., Pampanin, D.M., Naevdal, A., Oysaed, K.B., Gastaldi, L., Bechmann, R.K., 2008. Histopathology alterations and histochemistry measurements in mussel, *Mytilus edulis* collected offshore from an aluminium smelter industry (Norway). *Marine Pollution Bulletin* 57, 569-574.
- Abad, M., Ruiz, C., Martinez, D., Mosquera, G., Sanchez, J.L., 1995. Seasonal variations of lipid classes and fatty-acids in flat oyster, *Ostrea edulis*, from San Cibra (Galicia, Spain). *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* 110, 109-118.
- Axiak, V., George, J.J., Moore, M.N., 1988. Petroleum hydrocarbons in the marine bivalve *Venus verrucosa*: accumulation and cellular responses. *Marine Biology* 97, 225-230.
- Bancroft, J.D., 1967. An introduction to histochemical techniques. Butterworths, London.
- Beyer, J., Aas, E., Borgenvik, H.K., Ravn, P., 1998. Bioavailability of PAH in effluent water from an aluminium works evaluated by transplant caging and biliary fluorescence

- 264 measurements of Atlantic cod (*Gadus morhua* L.). Marine Environmental Research 46,  
265 233-236.
- 266 Bignell, J.P., Dodge, M.J., Feist, S.W., Lyons, B., Martin, P.D., Taylor, N.G.H., Stone,  
267 D., Travalent, L., Stentiford, G.D., 2008. Mussel histopathology: and effects of season,  
268 disease and species. Aquatic Biology 2, 1-15.
- 269 Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal  
270 parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel  
271 *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere 65, 913-921.
- 272 Brooks, S., Lyons, B., Goodsir, F., Bignell, J., Thain, J., 2009. Biomarker responses in  
273 mussels, an integrated approach to biological effects measurements. Journal of  
274 Toxicology and Environmental Health-Part a-Current Issues 72, 196-208.
- 275 Cajaraville, M.P., Abascal, I., Etxeberria, M., Marigomez, I., 1995. Lysosomes as  
276 cellular markers of environmental pollution: time dependent and dose dependent  
277 responses of the digestive lysosomal system of mussels after petroleum hydrocarbon  
278 exposure. Environmental Toxicology and Water Quality 10, 1-8.
- 279 Cancio, I., Ibabe, A., Cajaraville, M.P., 1999. Seasonal variation of peroxisomal enzyme  
280 activities and peroxisomal structure in mussels *Mytilus galloprovincialis* and its  
281 relationship with the lipid content. Comparative Biochemistry and Physiology C-  
282 Toxicology & Pharmacology 123, 135-144.
- 283 Da Ros, L., Moschino, V., Guerzoni, S., Halldorsson, H.P., 2007. Lysosomal responses  
284 and metallothionein induction in the blue mussel *Mytilus edulis* from the south-west  
285 coast of Iceland. Environment International 33, 362-369.
- 286 Domouhtsidou, G.P., Dimitriadis, V.K., 2001. Lysosomal and lipid alterations in the  
287 digestive gland of mussels, *Mytilus galloprovincialis* (L.) as biomarkers of  
288 environmental stress. Environmental Pollution 115, 123-137.
- 289 Garmendia, L., Soto, M., Cajaraville, M.P., Marigomez, I., 2010. Seasonality in cell and  
290 tissue-level biomarkers in *Mytilus galloprovincialis*: relevance for long-term pollution  
291 monitoring. Aquatic Biology 9, 203-219.
- 292 Gorbi, S., Lamberti, C.V., Notti, A., Benedetti, M., Fattorini, D., Moltedo, G., Regoli,  
293 F., 2008. An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*,  
294 for monitoring the impact of an offshore platform in the Adriatic sea. Marine  
295 Environmental Research 65, 34-49.
- 296 Hinton, D.E., Laurèn, D.J., 1990. Integrative histopathological effects of environmental  
297 stressors on fishes. American Fisheries Society Symposium 8, 51-66.
- 298 Koehler, A., 2004. Toxic injury and gender-specific hepatocellular carcinogenesis in  
299 flounder (*Platichthys flesus* (L.)). Aquatic Toxicology 70, 257-276.

- 300 Krishnakumar, P.K., Asokan, P.K., Pillai, V.K., 1990. Physiological and cellular  
301 responses to copper and mercury in the green mussel *Perna Viridis* (Linnaeus). Aquatic  
302 Toxicology 18, 163-173.
- 303 Krishnakumar, P.K., Casillas, E., Varanasi, U., 1994. Effect of environmental  
304 contaminants on the health of *Mytilus edulis* from Puget Sound, Washington, USA .1.  
305 Cytochemical measures of lysosomal responses in the digestive cells using automatic  
306 image-analysis. Marine Ecology Progress Series 106, 249-261.
- 307 Lowe, D.M., 1988. Alterations in cellular structure of *Mytilus edulis* resulting from  
308 exposure to environmental contaminants under field and experimental conditions.  
309 Marine Ecology Progress Series 46, 91-100.
- 310 Lowe, D.M., Moore, M.N., Clarke, K.R., 1981. Effect of oil on digestive cells in  
311 mussels: quantitative alterations in cellular and lysosomal structure. Aquatic Toxicology  
312 1, 213-226.
- 313 Lowe, D.M., Pipe, R.K., 1994. Contaminant induced lysosomal membrane damage in  
314 marine mussel digestive cells an *in-Vitro* study. Aquatic Toxicology 30, 357-365.
- 315 Marigomez, I., Baybay-Villacorta, L., 2003. Pollutant-specific and general lysosomal  
316 responses in digestive cells of mussels exposed to model organic chemicals. Aquatic  
317 Toxicology 64, 235-257.
- 318 Marigomez, I., Soto, M., Cajaraville, M.P., Angulo, E., Giamberini, L., 2002. Cellular  
319 and subcellular distribution of metals in molluscs. Microscopy Research and Technique.  
320 56, 358-392.
- 321 Marigómez, J.A., Angulo, E., Moya, J., 1986. Copper treatment of the digestive gland  
322 of the slug *Arion ater* L. 2. Morphometrics and histophysiology Bulletin of  
323 Environmental Contamination and Toxicology 36, 608-615.
- 324 Moore, M.N., 1988. Cytochemical responses of the lysosomal system and NADPH-  
325 ferrihemoprotein reductase in molluscan digestive cells to environmental and  
326 experimental exposure to xenobiotics. Marine Ecology Progress Series 46, 81-89.
- 327 Moore, M.N., 1990. Lysosomal cytochemistry in marine environmental monitoring.  
328 Histochemical Journal 22, 187-191.
- 329 Moore, M.N., Allen, J.I., 2002. A computational model of the digestive gland epithelial  
330 cell of marine mussels and its simulated responses to oil-derived aromatic  
331 hydrocarbons. Marine Environmental Research 54, 579-584.
- 332 Moore, M.N., Clarke, K.R., 1982. Use of microstereology and quantitative  
333 cytochemistry to determine the effects of crude oil-derived aromatic hydrocarbons on  
334 lysosomal structure and function in a marine bivalve mollusc, *Mytilus edulis*.  
335 Histochemical Journal 14, 713-718.
- 336 Moore, M.N., Simpson, M.G., 1992. Molecular and cellular pathology in environmental  
337 impact assessment. Aquatic Toxicology 22, 313-322.



- 338 Petrovic, S., Semencic, L., Ozretic, B., Ozretic, M., 2004. Seasonal variations of  
339 physiological and cellular biomarkers and their use in the biomonitoring of north  
340 Adriatic coastal waters (Croatia). *Marine Pollution Bulletin* 49, 713-720.
- 341 Pipe, R.K., Moore, M.N., 1986. An ultrastructural effect of phenanthrene on lysosomal  
342 membranes and distribution of the lysosomal beta-glucuronidase in digestive cells of the  
343 periwinkle *Littorina littorea*. *Aquatic Toxicology* 8, 65-76.
- 344 Regoli, F., 1992. Lysosomal responses as a sensitive stress index in biomonitoring  
345 heavy-metal pollution. *Marine. Ecology.-Progress. Ser.* 84, 63-69.
- 346 Regoli, F., Nigro, M., Orlando, E., 1992. Effects of copper and cadmium on the  
347 presence of renal concretions in the bivalve *Donacilla cornea*. *Comparative*  
348 *Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology* 102, 189-  
349 192.
- 350 Seed, R., 1976. Ecology. Marine mussels, their ecology and physiology, in: Bayne, B.L.  
351 (Ed.), *International Biological Programme 10*. Cambridge University Press, Cambridge,  
352 pp. 13-65.
- 353 Syasina, I.G., Vaschenko, M.A., Zhadan, P.M., 1997. Morphological alterations in the  
354 digestive diverticula of *Mizuhopecten yessoensis* (Bivalvia: Pectinidae) from polluted  
355 areas of Peter the Great Bay, Sea of Japan. *Marine Environmental Research* 44, 85-98.
- 356 Terman, A., Brunk, U.T., 2004. Lipofuscin. *International Journal of Biochemistry and*  
357 *Cellular Biology*. 36, 1400-1404.
- 358 Thompson, R.J., Ratcliffe, N.A., Bayne, B.L., 1974. Effects of starvation on structure  
359 and function in the digestive gland of the mussel (*Mytilus edulis* L.). *Marine Biology*  
360 *Association UK* 54, 699-712.
- 361 Tripp, M.R., Fries, C. R., Craven, M. A., Grier, C. E., 1984. Histopathology of  
362 *Mercenaria mercenaria* as an indicator of pollutant stress. *Marine Environmental*  
363 *Research* 14, 521-524.
- 364 Viarengo, A., 1985. Biochemical effects of trace metals. *Marine Pollution Bulletin* 16,  
365 153-158.
- 366 Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of  
367 biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced  
368 stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology C-*  
369 *Toxicology & Pharmacology* 146, 281-300.
- 370 Viarengo, A., Moore, M.N., Mancinelli, G., Mazzucotelli, A., Pipe, R.K., Farrar, S.V.,  
371 1987. Metallothioneins and lysosomes in metal toxicity and accumulation in marine  
372 mussels - the effect of cadmium in the presence and absence of phenanthrene. *Marine*  
373 *Biology* 94, 251-257.



374 Viarengo, A., Nott, J.A., 1993. Mechanisms of heavy metal cation homeostasis in  
375 marine invertebrates. *Comparative Biochemistry and Physiology C-Pharmacology*  
376 *Toxicology & Endocrinology* 104, 355-372.

377 Zorita, I., Ortiz-Zarragoitia, M., Soto, M., Cajaraville, M.P., 2006. Biomarker responses  
378 in mussels transplanted to a copper site gradient (Visnes, Norway). *Marine*  
379 *Environmental Research* 62, S398-S398.

380

381

## 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  $\mu$ m 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.

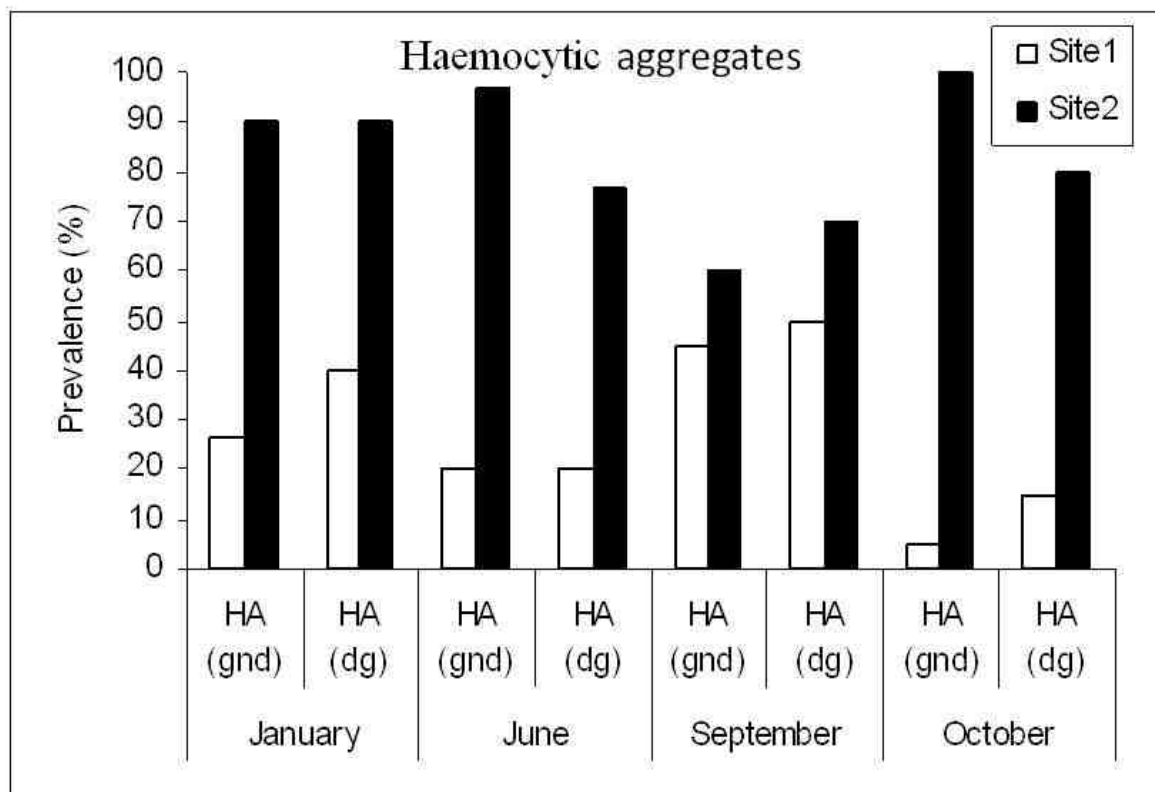


Figure1.

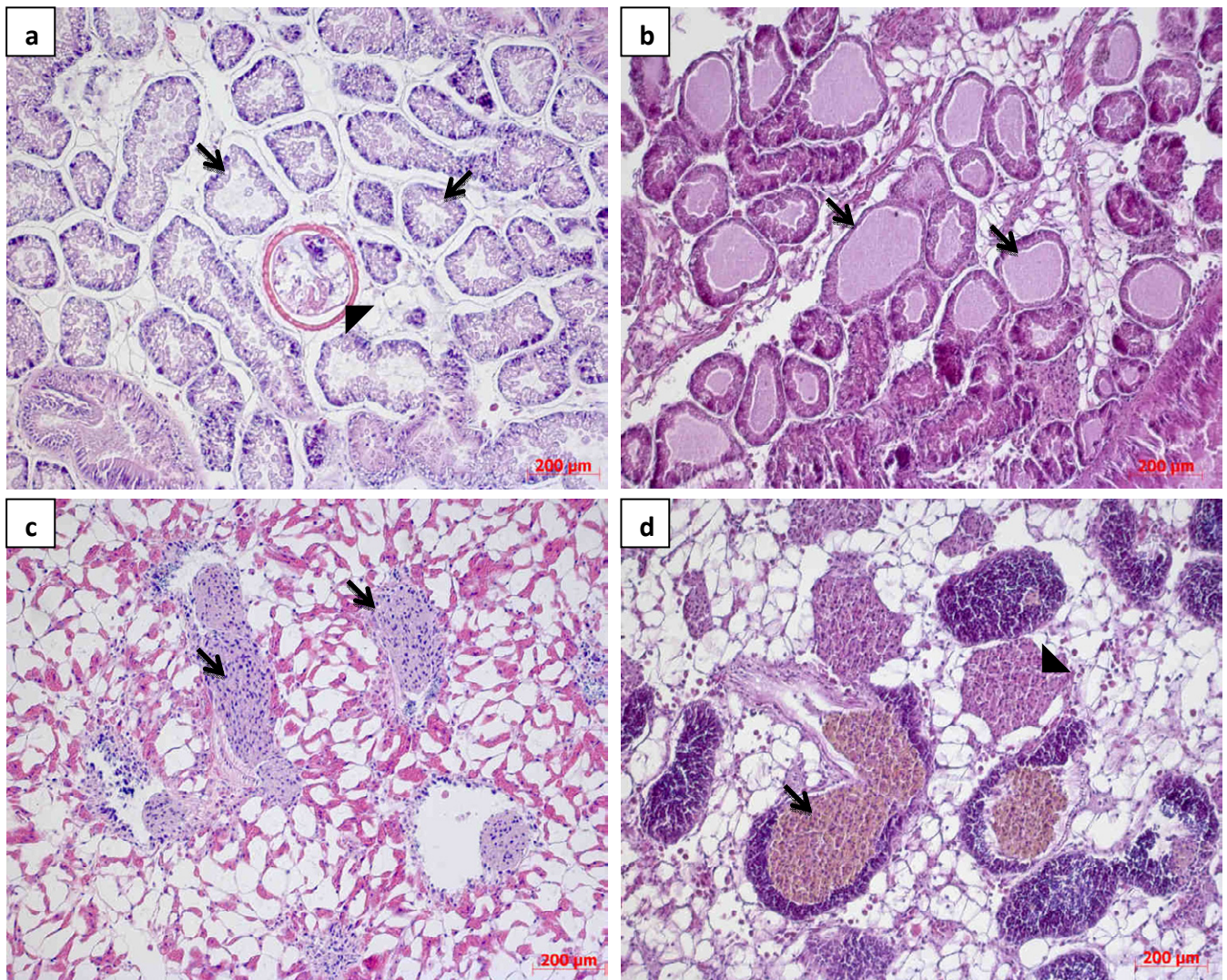


Figure 2

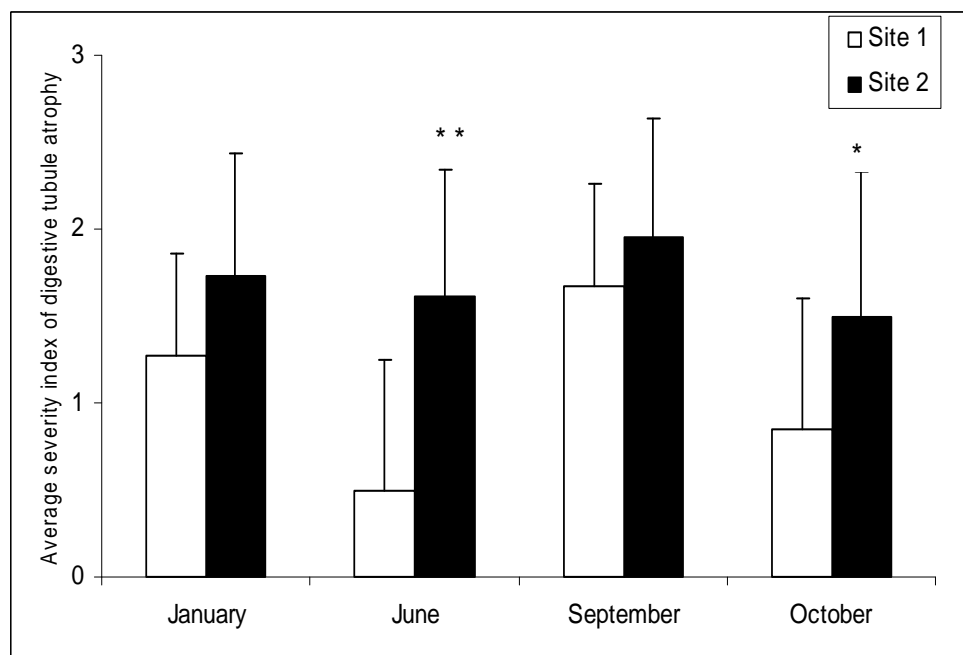


Figure3.



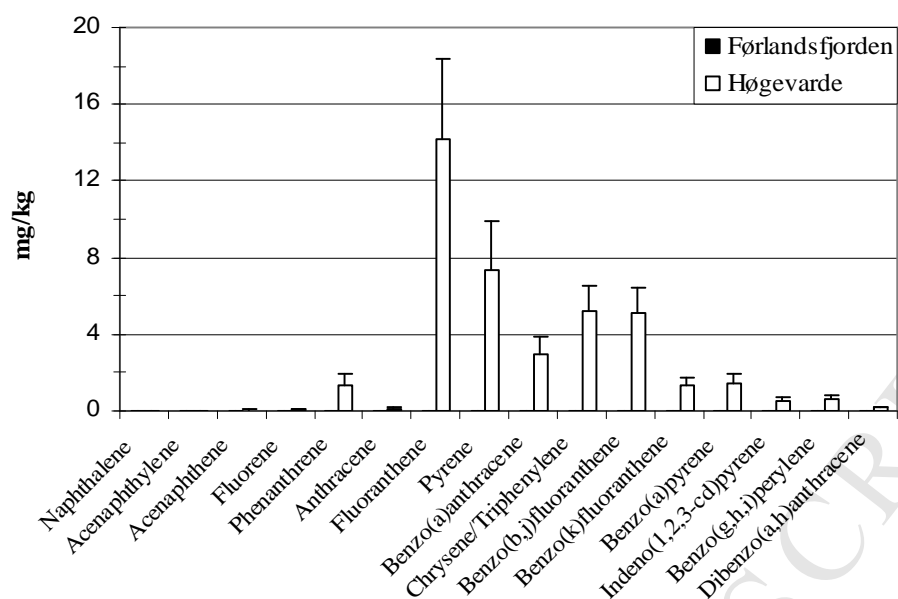


Figure 4. US EPA PAHs (in mg/kg wet weight given as average of six pools of 10 individuals from each locality) and st dev. The exposed locality Høgevarde is showing a pyrogenic PAH profile whereas levels in the reference locality Førlandsfjorden represents natural background levels. (Aarab *et al.*,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