



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, June, September and October 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.

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1. Introduction

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

Lipofuscin (LF) and neutral lipid (NL) are biomarkers of effects often used in biomonitoring programs. NL is also considered as an exposure biomarker against organic chemical compounds (Marigómez and Baybay-Villacorta, 2003). These parameters are

analysed in the digestive gland (DG) which can be affected by many pollutants (Axiak et al., 1988; Lowe et al., 1981; Lowe, 1988; Marigómez et al., 1986; Syasina et al., 1997; Tripp et al., 1984). The DG is the main site for metabolic activity; it is involved in immune defence, detoxification and in homeostatic regulation (Marigómez et al., 2002; Moore and Allen, 2002). The DG consists of many blind-ending tubules 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 (Cajarville 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. The present study is a follow up of this work and

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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 Karlsund. The site had a production of about 200,000 tons of aluminium per year, and the discharge of PAH to the Karlsund strait was approximately 450 kg annually (Fig. 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 during 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 and 30 specimens (6.0 cm ± 0.5 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% CaCl₂, 2, 5% NaCl) and dehydrated in alcohols. The tissues were cleared in methyl benzoate (C₆H₅CO₂CH₃), rinsed in benzene (C₆H₆) and embedded in paraffin. Histological sections (5 µm thickness) were cut using a microtome HM 355s (Microm, Bergman), mounted on slides, dried at 37 °C for 24 h and stained with haematoxylin 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 analysed 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 aluminium 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 [(FeCl₃)/(K₄[Fe(CN)₆])/H₂O (7.5:1:1.5)]. The sections were rinsed in 1% acetic acid (CH₃COOH) 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 ((C₂H₅)₃PO₄) 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 (400× 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® 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 (Fig. 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%).

3.1.3. Digestive gland pathology

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

3.1.4. Parasite infections

Three parasites were observed in mussels during the study: *Marteilia* sp., *Steinhausia mytilovum* and digenean metacercaria

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