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Integrated biological effects monitoring of the discharge of tailings from an iron ore mine using the mussel *Mytilus* species.

Running title: Biological effects monitoring of a mine discharge

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Abstract

The blue mussel (*Mytilus* spp.) has been used to assess the potential biological effects of the discharge effluent from the Sydvaranger mine, which releases its tailings into Bøk fjord at Kirkenes in the north of Norway. Metal bioaccumulation and a suite of sensitive health biomarkers were measured in mussels positioned for 6 weeks at varying distances from the discharge outlet. The biomarkers used included: stress on stress (SS); condition index (CI); cellular energy allocation (CEA); micronuclei formation (MN); lysosomal membrane stability (LMS), basophilic cell volume (VBAS); and neutral lipid (NL) accumulation. The individual biomarkers were integrated using the integrated biological response index (IBR/n). The accumulation of Fe was significantly higher in mussels located closer to the discharge outlet, indicating that these mussels had been exposed to the suspended mine effluent. The IBR/n results were in good agreement with the location of the mussels in relation to the distance from the discharge outlet and expected exposure to the mine effluent. The biomarker responses were not severe, but did exhibit effects for several of the biomarkers resulting in a higher IBR/n in the mussels within 3 km from the discharge outlet.

1. Introduction

Expanding mining activities, resulting from increasing societal demands for mineral resources, produces large volumes of waste in the form of processed tailings. In coastal areas such as Norway, such tailings may be disposed into fjords, which is a controversial approach. Whilst it is undeniable that negative effects on macrofauna communities appear near the discharge point where sedimentation rates are high (Allan, 1995). It is however, more unclear what the effects are when sedimentation rates and particle loadings related to the discharge are low and near ambient levels (Ramirez-Llodra et al., In Prep). In this paper marine mussels have been used to assess the potential biological effects of discharges of iron ore tailings. The study was designed to determine the sub-lethal biological effects of the tailing discharge on organisms living within the water column away from the physical disturbance of high particulate load. In order to achieve this aim, *Mytilus* spp. were positioned within the water column at known distances from the discharge outlet at Bøk fjord Norway, and a suite of sensitive biological effects endpoints and metal bioaccumulation measured.

The discharge of suspended tailings from Sydvaranger mine situated within the Arctic Circle in Kirkenes, Norway, was used in the field experiment. The mine is licenced by the Norwegian authorities to discharge up to 4 million tonnes of suspended mine tailings and 35 tonnes of flocculants annually into the nearby Bøk fjord. The flocculants polyDADMAC (Magnafloc LT38) and polyacrylamide (Magnafloc 10) are added to the tailings in order to assist in the removal of the iron ore, recycle freshwater and also to help aggregate the tailings prior to release into the fjord. The discharge of the treated tailing suspension proceeds via a submerged pipe line that extends 450 m out into the Bøk fjord from the Kirkenes harbour. The predominant effect on the local marine environment is through physical smothering of the

benthic fauna and flora within the immediate locality (50-100 m) of the discharge outlet (Berge et al., 2012).

Environmental monitoring studies using field transplanted mussels have been widely applied to assess the potential biological effects of a variety of point discharges on local marine environments including: urban effluents (Gagnon et al. 2006); produced water discharge from oil and gas activities (Hylland et al. 2008; Brooks et al. 2011); and mining (Zorita et al. 2006). Marine mussels are widely considered as one of the most suitable biological indicators of pollution for a number of reasons: 1) sessile filter feeders that accumulate contaminants in their tissues both through ingestion of particles and soluble substances in the water; 2) a recommended organism for biological effects monitoring; 3) have a wide array of sensitive biomarker tools available; and 4) are amenable to transplantation studies (ICES, 2011; Davies and Vethaak, 2012).

The biological effects measurements selected for this study were used to provide a sensitive evaluation of mussel health status with respect to exposure to the dissolved and particulate fraction of the mine tailings present within the water column. The suite of biological effects tools used included; lysosomal membrane stability (LMS) and micronuclei formation (MN) in haemocytes. The LMS (assayed as neutral red retention, NRR) assay is a well-established method that measures the functional integrity of lysosomes within cells (Livingstone et al. 2000; Moore et al. 2006). The MN test provides an indication of chromosomal damage, and has been found to show a time-integrated response to complex mixtures of pollutants (Baršienė et al. 2006; Gorbi et al. 2008). The frequency of MN is regarded as an important tool for *in situ* monitoring of DNA damage. Both LMS and MN are recommended as sensitive tools for mussel biomonitoring with validated assessment criteria available (ICES 2011; Davies and Vethaak, 2012).

Furthermore, the relative volume density of basophilic cells (VvBAS), neutral lipid accumulation (NL) and cellular energy allocation (CEA) where measured in the digestive gland tissue of the mussel. VvBAS provides a measure of the change in cell type composition from digestive cells to basophilic cells, which is known to occur following exposure to environmental contaminants (Marigómez et al. 2002; Zaldibar et al. 2007).

The lysosomal storage of neutral lipids in mussel digestive glands has been identified as a useful marker of change in cellular physiology (Viarengo et al. 2007). Elevated levels of neutral lipid within the lysosomes of digestive glands of mussels have been linked with organism stress and reduced health status. The CEA approach measures the metabolic resources by quantifying the available energy reserves and energy consumption at a cellular level of biological organisation and incorporates all components into a net cellular energy budget of the organism (De Coen and Janssen, 1997; Erk et al. 2011). The net energy budget provides a measure of stress in an organism brought about by environmental pressures. The CEA has been applied in a variety of aquatic field and laboratory studies using different organisms including Crustacea and Mollusca (Smolders et al. 2004; Olsen et al. 2007).

In addition to the cellular biomarkers within the haemocytes and digestive gland, whole organism responses such as condition index (CI) and stress on stress (SS) were measured. The CI provides a simple measure of organism health status, encompassing physiological activity such as growth, reproduction, secretion, etc., under environmental conditions. The SS measures the ability of the mussel to survive out of water. The test assumes that mortality in air would occur more rapidly in pre-stressed animals than in control animals and is a whole organism response providing a relative indication of the individual mussel's health status (Hellou and Law, 2003; Viarengo et al. 1995). Both CI and SS provide a measure of the

general health of the mussel and together with the suite of biomarkers provide a holistic and integrative approach to biological effects studies.

The overall objective of the study was to provide an assessment of the potential biological effects of the discharge water from the Sydvaranger mine within the receiving waters of Bøkfjord. The potential impact of the discharge was assessed using an integrated suite of biomarker tools in mussels held at known distances from the discharge outlet. In addition metal body burden data was also included to support the biological effects endpoints.

2. Methods

2.1. Collection and deployment of field mussels

Mussels (*Mytilus* spp.) were collected from Brashamn (N 69 53.966 E 29 44.723), an area approximately 40 km from the study site considered to be a clean location unaffected by known anthropogenic inputs. The *Mytilus* species collected were considered to be mostly composed of *M. edulis*, with previous reports identifying populations within the area containing approximately 80% *M. edulis* (Brooks and Farmen, 2013). However, since species identification was not confirmed the mussels used in this study will be referred to as *Mytilus* spp. only.

The collected mussels (length 5.74 ± 0.68, mean ± SD) were gently cleaned of excess debris with seawater and transplanted into the field in soft nylon mesh socks. Care was taken to ensure that the mussels had sufficient space within the mesh so as not to impede filtration. Approximately 80 mussels were attached to three moorings positioned at known distances (0.6, 3 and 10 km) from the discharge outlet (Groups 1, 2 and 3 respectively, Figure 1). Turbidity measurements showed that the discharged tailings generally do not enter the euphotic part of the water column. The mussels were therefore held at a depth of 30 m. The mussel cages were deployed in September and retrieved after approximately 6 weeks. Turbidity measurements performed during the mussel deployment revealed that group 1 mussels were exposed to particles from the tailings (1.5 - 3 FTU). In contrast mussel groups 2 and 3 were not significantly impacted by particulates from the discharge (<0.5 FTU). Mussel group 3, were considered a field control group, which experienced the stress of transplantation but were unaffected by the mine effluent.

The mussels from groups 1-3 were retrieved after 6 weeks and additional mussels (group 4) were collected from the same location as those used in the field exposure groups. The collected mussels were placed in a cooler box for transport back to the field laboratory in Kirkenes. The mussels were processed immediately after collection, with all mussels sampled within 1 h of collection. There were no mortalities observed upon collection for any of the exposure groups.

Where possible, the same mussel was used to measure a number of biological endpoints. In the first 20 mussels, LMS (as NRR) was measured alongside VvBAS, and NL. A further 20 mussels were used for MN and CEA. A further 20 mussels were used for SS, whilst the remaining mussels were used for metal analysis. Biometry (length, weight) was recorded in all mussels sampled.

2.2. Tissue chemistry

For each exposure group, triplicate mussel samples were collected for analysis of selected metals. Pooled mussel samples of ten individuals were removed from their shells and placed in high temperature treated (560°C) glass containers. The mussels were immediately frozen on dry ice and transported to NIVA, Oslo. All samples were stored at -20°C until required for analysis. Metal concentrations were determined in homogenised whole mussel samples using inductively coupled plasma-mass spectrometer (ICP-MS, Perkin-Elmer Sciex ELAN 6000).

2.3. Biomarkers

2.3.1. Condition index

The condition index was measured on twenty mussels from each group by determining the ratio of the wet weight of the soft tissue to the total weight (shell + soft tissues + palleal liquid) of the mussel, multiplied by 100 (Damiens et al. 2007).

$$CI = \left(\frac{Soft\ tissue\ weight\ (g)}{Total\ weight\ (g)}\right) \times 100$$

2.3.2. Stress on stress

Twenty mussels were selected from each group and placed in a humid incubator at 15 ± 0.5ºC. Mussel mortality was checked every 24 ± 4 h and mortalities were recorded until all mussels had died. Mussels were considered dead if their shells were gaping and showed no sign of movement when gently tapped on the shell.

2.3.3. Cellular energy allocation

Total available energy (E_a) was calculated by combining the energy sources from carbohydrate, lipid and protein. CEA calculations were made by comparison of the available energy (E_a) and consumed energy (E_c) using the equation of Verslycke and Janssen (2002) with some modifications (Erk et al. 2008). The w.w. denotes the wet weight of the mussels in grams (g).

$$E_a = E_{carbohydrate} + E_{lipid} + E_{protein} \text{ (mJ/ mg w.w.)}$$

$$Ec = \text{Electron Transport System (ETS) activity (mJ/ mg w.w. / h)}$$

$$CEA = E_a / E_c$$

Digestive gland tissue was diluted eleven fold with homogenisation buffer (0.1 M Tris-HCl buffer, 0.4 M $MgSO_4$, 15% polyvinylpyrrolidone and 0.2% Triton X-100, pH 7.5), homogenised and aliquoted. The ETS analysis was carried out directly after homogenisation and protein, carbohydrate and lipid samples were frozen and stored at -80ºC until further analysis.

Energy consumption, measured as ETS activity was determined by pipetting 100 µL 0.1 M BSS (Trizma HCl/ base buffer pH 7.5, 0.3% Triton X), 50 µL NAD(P)H solution (1.17 nM NADH, 250 nM NADPH in distilled water), 50 µL of sample and 100 µL iodonitrotetrazolium chloride (INT) in wells of a 96 well plated. Four replicate measurements were made for each sample on a spectrophotometer (Molecular Devices Thermomax plate reader, Sunnyvale, USA) at 490 nm, 2ºC, every 15 sec for 10 min). V_{max} was calculated by the Softmax Pro Software (Molecular Devices, Sunnyvale, USA).

Protein concentration was determined using the BioRad DC protein assay reagents (Hercules, California, USA) as described by Lowry et al. (1951).

Carbohydrate was measured in samples that were first washed with 15% trichloroacetic acid (TCA) and centrifuged (18.8 g for 5 min). The resulting pellet was washed with 5% TCA and centrifuged again (18.8 g for 5 min). The supernatants from the two centrifugation steps were mixed and carbohydrate measured spectophotometrically at 490 nm after the addition of 1 part phenol and 4 parts sulphuric acid. Glycogen from bovine liver was used as a standard.

Lipid measurements were based on the technique described by Bligh and Dyer, (1959). Samples containing 200 µL were added to 500 µL chloroform. After vortex mixing, 500 µl methanol and 250 µL distilled water were added and the solution was mixed further and centrifuged (18.8 g for 5 min). The chloroform phase was then removed and added to tubes

containing 500 µL sulphuric acid and incubated at 200ºC for 15 minutes. Samples were cooled to room temperature before the addition of 1 ml distilled water and absorbance measured at 340 nm (Perkin Elmer Victor 1420, Massachusetts, USA). Two reference samples were used for each plate and four replicate measurements were made for each sample. Lipid concentrations were calculated through the use of a tripalmitine standard dilution sequence.

2.3.4. Lysosomal membrane stability

Lysosomal membrane stability was measured in mussel haemocytes using the Neutral Red Retention (NRR) procedure adapted from Lowe and Pipe (1994). Approximately 0.1 ml of haemolymph was sampled from the adductor muscle of the mussel with a syringe containing approximately 0.1 ml of physiological saline (pH 7.2). The haemolymph/saline solution was gently mixed in a microcentrifuge tube, from which a 40 µl sample was pipetted onto the centre of a microscope slide. The slide was left in a dark humid chamber for 15 min to allow the cells to adhere to the slide. Following incubation, excess liquid was gently removed from the slide and 40 µl of neutral red solution (Sigma) was added and a cover slip applied. The neutral red solution was taken up inside the haemocytes and stored within the lysosome. The ability of the lysosome to retain the neutral red solution was visually inspected every 15 min for 1 h and then every 30 min for 2 h by light microscopy (x40 objective). The test was terminated and the time recorded when greater than 50% of the haemocytes leaked the neutral red dye out of the lysosome into the cytosol.

2.3.5. Micronuclei formation

Approximately 0.1 ml of haemolymph was collected from the posterior adductor muscle of each mussel with a hypodermic syringe containing 0.1 ml of PBS buffer (100 mM PBS, 10 mM EDTA). The haemolymph and PBS buffer were mixed briefly in the syringe and placed on a microscope slide. The slide was then placed in a humid chamber for 15 min to enable the haemocytes to adhere to the slides. Excess fluid was drained and the adhered haemocytes were fixed in 1% glutaraldehyde for 5 min. Following fixation, the slides were gently rinsed in PBS buffer and left to air-dry overnight. The dried slides were stored in the dark at room temperature until further analysis.

Slides were stained with 1 μg/ml bisbenzimide 33258 (Hoechst) solution for 5 min, rinsed with distilled water and mounted in glycerol McIlvaine buffer (1:1). The frequency of MN was measured on coded slides without knowledge of the exposure status of the samples to eliminate bias. The frequency of MN in haemocytes was determined microscopically with a ×100 objective. A total of 2000 cells were examined for each experimental group of mussels.

Only cells with intact cellular and nuclear membrane were scored. MN were scored when: 1) nucleus and MN have a common cytoplasm, 2) colour intensity and texture of MN is similar to the nucleus, 3) the size of the MN is equal or smaller than 1/3 of the nucleus, 4) MN are apparent as spherical structures with a sharp contour.

2.3.6. Volume of basophilic cells

Digestive glands were sampled from individual mussels and snap frozen in liquid nitrogen. The preserved tissue was stored at -80ºC until sectioned on a cryostat. Cryostat sections (10 μm) were mounted on slides and fixed in Baker's calcium formol for 5 min. Fixed slides were rinsed in distilled water and stained with Gills haematoxylin for 15 secs. After this time they

were rinsed in flowing tap water for 20 min and stained with Eosin-Phloxin solution for 30 seconds, before rinsed in 80% ethanol. The stained slides were mounted with Euparal medium and left to dry overnight before microscopic examination.

As an indication of whether cell-type replacement occurred, the volume density of basophilic cells (VvBAS) in the digestive gland of mussels was determined microscopically by means of stereology using a Weibel graticule eye piece (M-168; Weibel, 1979). Counts were made in 3 fields of 2 different sections (20 to 40 μm apart) of the mussel digestive gland (×400 magnification). The volume density of basophilic cells (VvBAS) was calculated using the equation:

$$VvBAS = \frac{X_1 + X_1 \ldots + X_n}{m \times n}$$

Where X = number of segments edges (from Weibel graticule) falling on basophilic cells; m = total number of segment edges falling on digestive tissue; n = number of counts (6 for each mussel).

2.3.7. Neutral lipid accumulation

Digestive gland sections (10 μm) were prepared on a cryostat and mounted on to microscope slides where they were fixed in Bakers calcium formol for 15 min. The sections were briefly rinsed in distilled water before placed in 60% triethyl phosphate for 1 min, and stained in oil red O solution for 15 min at room temperature. After the 15 min staining, sections were washed in 60% triethyl phosphate and then rinsed in distilled water. Slides were left to air dry before mounted in glycerol gelatin.

2.4. Integrated biological response index

The Integrative Biological Response (IBR) index was developed to integrate biochemical, genotoxicity and histochemical biomarkers (Beliaeff and Burgeot, 2002). In the present study CI, SS, NL, NRR, VvBAS, CEA and MN were the biomarkers selected for the IBR calculation. The inverse values of CI, SS, NRR and CEA were used since a decrease was reflective of adverse impact. The calculation method is based on relative differences between the biomarkers in each given data set. Thus, the IBR index is calculated by summing-up triangular star plot areas (a simple multivariate graphic method) for each two neighbouring biomarkers in a given data set, according to the following procedure:

1. Calculation of the mean and standard deviation for each sample.
2. Standardisation of the data for each sample: xi' = (xi-x)/ s; where, xi' = standardised value of the biomarker; xi = mean value of a biomarker from each sample; x = general mean value of xi calculated from all compared samples (data set); s = standard deviation of xi calculated from all samples.
3. Addition of the standardised value obtained for each sample to the absolute standardised value of the minimum value in the data set (yi = xi' + |xmin'|).
4. Calculation of the Star Plot triangular areas by multiplication of the obtained standardised value of each biomarker (yi) with the value of the next standardised biomarker value (yi + 1), dividing each calculation by 2 (Ai = (yi * yi + 1)/ 2).
5. Calculation of the IBR index which is the summing-up of all the Star Plot triangular areas (IBR=∑Ai) (Beliaeff and Burgeot, 2002). Since the IBR value is directly dependent on the number of biomarkers in the data set, we divided the obtained IBR value by the number of biomarkers used in each case (n=7) to calculate IBR/n, according to Broeg and Lehtonen (2006).

2.5. Statistical analysis

Analysis of variance (ANOVA) followed by a Tukey post-hoc test was performed on the biological effects data to determine statistical differences between groups. Homogeneity of variance was determined with a Levene's test prior to testing, and where necessary, data were log transformed to achieve homogeneity. However, in cases where homogeneity of variances was not achieved a Kruskal-Wallis non-parametric analysis was used. The level of significance was set to $p=0.05$.

3. Results

3.1. Metal concentrations in mussel tissues

The results of the metal analysis of whole mussel homogenates of the four groups are shown in figure 2. Of the ten metals measured only aluminium (Al), iron (Fe) and lead (Pb) showed significant differences in concentration between the exposure groups. Mussels from group 1 (79.3 ± 12.6 mg/ kg w.w.), located 0.6 km from the outlet, had significantly higher Fe concentration than all the other mussel groups (2: 3 km; 3: 10 km; 4 source population) (ANOVA, Tukey, $p<0.05$). Fe concentrations from group 1 were over 5 times the concentration measured in mussels from the field reference group (group 3, 14.67 ± 2.1 mg/ kg w.w.). Concentrations of Al were significantly higher in group 1 and 2 mussels compared to mussels from group 3 (ANOVA, Tukey, $p<0.05$). In contrast, Pb was significantly elevated in mussels from group 2 and 3 compared to the source mussels from group 4.

3.2. Biomarkers

3.2.1. Condition index

The mean CI of mussels closest to the discharge outlet (group 1) was significantly lower than those from the other two field groups (groups 2 and 3, $p<0.05$, Fig. 3**Feil! Fant ikke referansekilden.**). However, the mean CI of mussels from the source population (group 4) was comparable to those mussels closest to the discharge outlet (group 1).

3.2.2. Stress on Stress

The stress on stress test showing the survival time in air revealed clear differences between the mussel groups (Fig. 4**Feil! Fant ikke referansekilden.**). Longer survival durations were observed in mussels from groups 2 and 3 compared to groups 1 and 4. The duration resulting in 50% mortality (LT50) was lowest in mussels from group 4 and group 1, approximately 8 days and 9 days respectively, whilst LT50 increased to 11.5 days and 12 days for group 2 and group 3 respectively.

3.2.3. Cellular Energy Allocation

The results of the CEA analysis are presented in table 1**Feil! Fant ikke referansekilden.**. Lipid, proteins and carbohydrates each converted into their energetic equivalent, enabled comparison of the relative contribution of these fractions to the mussels overall energy budget. Protein was by far the most important energy source with average contribution of 70-75% of the overall energy budget in all mussel groups. Lipid and carbohydrate made up the remaining 25-30%. Although carbohydrates appeared to be more important in mussels from group 1, there were no significant differences between the exposure groups. This was also true for lipids and proteins. Furthermore, there were no significant differences between the available energy (Ea), the energy consumed (Ec) or the calculated CEA for any of the exposure groups.

3.2.4. Lysosomal membrane stability

Lysosomal membrane stability (LMS) measured as NRR, showed differences between the exposure groups (Fig. 5**Feil! Fant ikke referansekilden.**). Significantly lower NRR was

measured in mussels from group 2 compared to group 4 ($p<0.05$). However, for the field exposed mussels (groups 1-3) no significant differences in NRR were observed.

3.2.5. Micronuclei formation

There were no significant differences found between the numbers of MN in the mussels of the different exposure groups (Fig. 6**Feil! Fant ikke referansekilden.**). Mean MN frequencies ranged between 3.6 and 4.7 per 1000 cells.

3.2.6. Volume of basophilic cells

The volume of basophilic cells (VvBAS) in the digestive gland tissue was significantly higher in mussels from group 1 (0.21 ± 0.02 $\mu m^3/\mu m^3$, Fig. 7) compared to all other groups (ANOVA, Tukey, $p<0.05$). Mussels from group 2 (0.16 ± 0.03 $\mu m^3/\mu m^3$) were significantly higher than mussels from group 4. The mussels from groups 3 and 4 had VvBAS values below 0.12 $\mu m^3/\mu m^3$.

3.2.7. Neutral lipid accumulation

Neutral lipid accumulation was significantly higher in mussels from group 2 compared to the source mussels (group 4, Fig. 8) (ANOVA, Tukey, $p<0.05$). No significant difference was found between mussels from the field exposed groups (groups 1-3).

3.3. Integrated biological response

The IBR/n index was calculated from star plots of normalised data from all seven biomarker endpoints (Fig. 9). A higher IBR/n value corresponds to increased stress and impaired health on the mussels. The inverse mean value of CI, NRR, CEA and SS was used in the calculation,

since for these endpoints a higher mean value indicates good health. The lowest IBR/n was observed in mussels from groups 3 and 4, which correspond to the field exposed reference and source population mussel groups. The highest IBR/n was calculated in the biomarker responses from mussel groups 1 and 2, which were the caged mussels located approximately 0.6 km and 3 km from the discharge outlet (IBR/n =1.1-1.2).

4. Discussion

4.1. Chemical exposure

The metal analysis showed a clear signal for Al and particularly Fe in the mussel tissues, with mussels from the closest stations having significantly higher Fe body burden concentrations, decreasing with distance away from the discharge outlet. Since Sydvaranger is an iron ore mine, the detection of elevated Fe in the Bøk fjord may be expected. The Fe body burden concentration in the mussels in addition to the higher turbidity recorded at the closest station confirms that the mussels had been exposed to the mine discharge plume. Therefore, it is suggested that the biological effects observed in the mussels from the two closest exposure groups were at least in part caused by the exposure to the mining tailing effluent. The relatively low concentration of Fe in the mussels positioned 10 km (group 3) from the mine tailing outlet indicates that these mussels were not significantly exposed to the mining effluent. This was also the case for all of the other metals analysed, which suggests that the mussels from this group could be used as a suitable reference when comparing the biological effects data.

4.2. Biological responses

The Fe body burden data indicated that only mussels positioned at the two closest exposure groups (0.6 km and 3 km) from the discharge outlet were exposed to the mining effluent. While the biomarker results overall appeared to agree with this, individual biomarkers were found to have varying responses.

The CI provides important information on the physiological status of the mussel, of which detrimental effects may be caused by either general environmental pressures, such as food availability, seasonal cycle, and/ or chemical exposure. In the mussels caged at Bøk fjord, the CI was able to differentiate between mussels from group 1 and the other two caged mussels (groups 2 and 3). The reduction in the CI of mussels from group 1 corresponded well with the relative distance from the discharge outlet and may be partial explained in terms of exposure to the mine effluent. However, mussels collected from the source population (group 4), had an equally low CI as mussels from group 1, which suggest that the field mussels, taken from the same population as the transplanted groups were experiencing a similar degree of physiological stress. Since contaminant exposure was unlikely in the field reference station, it is possible that food availability may be responsible. However, the CI value in the Bøk fjord mussel groups was almost double that of transplanted mussels from a similar study where the same method of measuring CI was used (Damiens et al. 2007). The relatively higher CI in the Bøk fjord mussels suggests that these individuals were not starved of food, but differences in food availability were sufficient enough to discriminate between the groups.

The ability of the mussel to survive in air provides a measure of the physiological status. As described above for CI, the physiological status of the whole mussel can be influenced by environmental pressures such as food availability and/or chemical exposure as well as habitat adaptation (e.g. intertidal). The results of the SS were highly similar to CI with reduced

survival times in exposure groups 1 and 4 compared to groups 2 and 3. Since these biomarkers are closely related, the factors influencing survival are likely to be similar to those that influence CI and vice versa. In comparison to other field studies were SS measurements have been taken, mean LT50 values of 9 days were recorded in reference mussels *(Mytilus* spp.) from an intertidal habitat on the south east coast of England, reducing to 7 and 5 days in mussels influenced by anthropogenic sources (Hellou and Law, 2003). Furthermore, LT50 values ranged from 150 h (6.25 days) to 80 h (3.3 days) in *Mytilus edulis* collected from the coastal waters of the Scheldt estuary, whilst the LT50 reduced to around 55 h (2.3 days) in mussels transplanted into cages within the Scheldt estuary (Wepener et al. 2008). When taking these studies into prospective, the mussels within Bøkfjorden with an LT50 of 12 to 8 days would indicate mussels of a relatively good level of physiological status.

Cellular energy allocation is a physiological biomarker providing information on the metabolic processes of the organism. In the mussel groups from Bøk fjord there were no significant differences in any of the CEA parameters between the groups, indicating no exposure effects due to the mining effluent. From the energy constituents, protein was by far the largest, contributing 70-75% of the available energy supply, with carbohydrates and lipids sharing the remaining 25-30%. Low to intermediate levels of contaminant exposure are known to promote proteins due to the detoxification processes, whilst low levels of carbohydrate and lipids could suggest lack of food availability (Erk et al. 2011). However, since no differences occur between the exposure groups these explanations are not likely to be responsible in the mussels from this study. Overall, the CEA value ranging between 500 to 550 for all exposure groups, implies that the mussels had a positive energy budget, using less energy than they had available, which would suggest that all groups were in reasonably good health.

The use of NRR for the measurement of LMS is one of the most widely used biomarkers in aquatic monitoring. Partly due its widespread use there has been numerous field and laboratory study data collected for *Mytilus* spp. that has been used to compile various assessment criteria (ICES, 2011). From these assessment criteria mussels are considered in good health if there NRR is above 120 min, stressed but compensating if between 120 and 50 min and severely stressed and probably exhibiting pathology if the value is below 50 min. Mean NRR of the mussel groups from Bøk fjord were between 60 and 110 min and therefore fall into the category described as stressed but compensating. Even the mussels collected from the source reference group (group 4) did not have a mean NRR above 120 min and were thus considered to be experiencing stress. There was no significant difference between the exposure groups (groups 1 to 3) although generally they had NRR values lower than the source reference group (group 4). This may be the result of the additional stress on the mussels through transplantation into a new environment.

The mean MN frequency in the exposure groups was within a relatively narrow range from 3.6 to 4.7 per 1000 cells with no significant differences between the groups. However, suggested assessment criteria for MN have been established using data available on studies of mussels from the North Sea, Northern Atlantic and the Mediterranean, calculating the empirical 90 percentile as background/threshold level of MN incidences (ICES, 2012). From this report, based on over 600 data points, background levels in field transplanted *M. edulis* for 4 to 6 weeks were calculated as 4.06 MN per 1000 cells, with MN incidence above 4.06 considered as an effect response. Based on this assessment criteria, mussel from groups 1 and 2 had an MN above this threshold, which may be considered to demonstrate an effect response, whilst mussel groups 3 and 4 were within background levels.

Digestive cell loss, measured as VvBAS in the digestive gland is considered a sensitive indicator of general stress in marine mussels (Zaldibar et al. 2007). The VvBAS values below 0.10 $\mu m^3/\mu m^3$ have been considered to indicate a healthy condition; whereas VvBAS values higher than 0.12 $\mu m^3/\mu m^3$ indicate a stress situation (Marigómez et al. 2006). The mussels of exposure groups 1 and 2 had a VvBAS above the 0.12 $\mu m^3/\mu m^3$ threshold, which clearly indicate a stress response, whilst those from groups 3 and 4 were between the 0.12 and 0.10 $\mu m^3/\mu m^3$ suggesting some stress above typical background. However, it should be pointed out that these threshold levels were mostly based on native field mussel populations in the Adriatic/ Mediterranean area and likely to differ slightly to those of caged mussels located within the Arctic Circle, although to what extent is currently not known.

The accumulation of NL within the digestive gland cells of mussels is considered to be a stress response particularly for organic chemicals (Lowe and Clarke, 1989, Cajaraville et al. 1992). In our study no significant differences were found between NL of field exposed mussels with distance from the mining effluent. However, differences were found between the source reference group and the caged mussel groups, which may suggest that the difference between the groups may not be related to chemical exposure but other sources of environmental stress. Currently no assessment criteria are available for lipofuscin accumulation in field mussels.

4.3. Integrated biological response

The integrated biological response (IBR/n) was used to provide a visual integration of all seven biomarkers, showing the contribution of each individual biomarker to the group IBR/n score. Higher IBR/n scores indicate increased biological response and poorer health status of the mussel. The position of the biomarkers within the star plots is particularly important since

they can provide different IBR/n scores with different arrangements. For this reason it was important that the biomarkers that measure similar physiological and/or cellular functions are grouped together. In the present study: SS, CI and CEA measured general physiology and metabolism; NL, VvBAS and NRR measured cellular responses; and MN measured genotoxicity in the mussels, these biomarkers were grouped together accordingly.

The lowest calculated IBR/n was in the two reference groups (3 and 4) with IBR/n of 0.17 and 0.29 respectively. For the source reference (group 4), the largest biomarker contributions to the IBR/n were from the physiological biomarkers, SS and CI. These responses were unlikely to be due to chemical exposure but environmental factors such as food availability and competition. The highest IBR/n of 1.25 and 1.15 were recorded in exposure groups 2 and 1 respectively. However, the biomarkers contributing to these respective scores were quite different. For mussels in group 1, CI, SS and VvBAS were the main contributors, whilst those contributing for group 2 were MN, NRR, NL and CEA.

Interpretation of the IBR/n should be performed with some caution. For example, the MN data for the different groups revealed very little difference between the groups. However despite this, the standardised biomarker values on the star plots were much higher in group 2 and 1 than 3 and 4. A similar phenomenon was observed for CEA with groups 2 and 3 receiving the higher contributions. Broeg and Lehtonen (2006), have highlighted that the IBR/n is an oversimplification of very complex exposure situations within the field, and that the IBR/n result should not be taken at ”face value” but rather as a tool to direct further actions. With this in mind it appears that mussels from groups 1 and 2 were the most affected, although an IBR/n of 1.2 would suggest only relatively low biological effects, particularly when looking at the individual biomarker responses behind these calculated indices.

Whether the effects exhibited by the mussels are a result of exposure to the mine effluent alone are not entirely clear since other sources of contaminant input into Bøk fjord are known to exist in the vicinity of Kirkenes. For example, sediments in the Kirkenes harbour area are reported to be contaminated with elevated concentrations of copper, polycyclic aromatic hydrocarbons (PAH) and tributyltin (TBT) from boat-yard operations (Norconsult, 2008). In the present study, copper analysis of the mussel tissue did not indicate any significant bioaccumulation that could be attributed to exposure from the contaminated sediment, although TBT and PAH concentrations were not measured.

An addition source of contaminant input in to the fjord is from the local discharge of untreated sewage in the surface waters of the harbour area. It is however unclear whether there is any influence of the sewage discharge at the depth where the mussels were located (i.e. 30 m). Although it is anticipated that the sewage will mainly be restricted to the surface waters and not directly influence the mussels, the effects of exposure to sewage related chemicals such as endocrine disruptors cannot be completely excluded.

In the light of increasing mining activities worldwide, and the problems associated with land-disposal, there is a need for further research which adequately addresses the physical, chemical, and biological aspects of the effects of marine disposal of mine tailings (Ramirez-Llodra, et al. In Prep). In this regard the use of a well described monitoring organism such as the blue mussels, together with an integrated biomarker approach as described in the present study, may be advantageous in elucidating the ecological effects other than direct smothering.

5. Conclusions

The result indicates that tailings disposal may cause slight, but significant biological effects in part of the recipient up to 3 km from the discharge. The Fe body burden was significantly

higher in mussels at the two closest exposure groups (0.6 km and 3 km), and particularly at the closest exposure group, indicating that these mussels had been exposed to the discharge effluent from the Sydvaranger mine. Exposure to the mine tailings may have contributed to the impaired health of the mussels positioned at the two closest locations, although other sources of contaminants within Bøk fjord have been previously identified and cannot be ruled out. Overall, the IBR/n results were in good agreement with the location of the exposure groups in relation to the distance from the mine discharge outlet. The biomarker responses were not severe but did exhibit effects for several of the biomarkers, resulting in a higher IBR/n in the mussels closest to the outlet.

6. Acknowledgements

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Captions

Table 1. The available energy (E_a) in the digestive gland of mussels calculated from the different fractions: lipid, protein and carbohydrate. The Ea divided by the energy consumed (E_c) was used to calculate the cellular energy allocation (CEA) (mean ± SE, n=15). No statistical significant differences between the groups.

Fig. 1. Approximate locations of the three field mussel groups in the Bøk fjord in relation to the Sydvaranger mine discharge outlet. Approximate distances from the outlet: group 1 – 0.6 km (N69 44.399 E30 02.489); group 2 – 3 km (N69 45.585 E30 04.159); group 3 – 10 km (N69 49.142 E30 05.938). Group 4 – 40 km (source of the mussels, N69 53.966 E29 44.723).

Fig. 2. Metal concentrations in whole mussel homogenates. Groups 1- 3 represent field mussels, group 4 represents the wild source population (mg/ kg w.w., median ± quartiles, n=3). Values with different letters (a, b, c) are significantly different from each other ($p<0.05$). Ba and Co were below the limit of detection (LOD) of 0.2 and 0.1 mg/kg w.w. respectively and not shown.

Fig. 3. Condition indices of mussels from the different exposure groups (mean ± standard error (box), standard deviation (outer lines)). Groups 1- 3 represent field mussels at 0.6, 3 and 10 km from the discharge outlet respectively, group 4 represents the wild source population. Groups labelled with different letter are significantly different from each other (ANOVA, Tukey, $p<0.05$, n=20).

Fig. 4. The percentage survival of mussels over time from the different groups exposed to air at 15 ± 0.5oC (n=20). Groups 1- 3 represent field mussels at 0.6, 3 and 10 km from the discharge outlet respectively, group 4 represents the wild source population.

Fig. 5. Lysosomal membrane stability measured as neutral red retention time (NRR) in the lysosomes of mussel haemocytes from the different groups. Groups 1- 3 represent field mussels at 0.6, 3 and 10 km from the discharge outlet respectively, group 4 represents the wild source population. Mean, standard error (box) and standard deviation (outer line). Groups labelled with the same letter are not significantly different from each other (Kruskal Wallis ANOVA, $p<0.05$, n=20).

Fig. 6. Micronuclei formation in mussel haemocytes from the different groups. Groups 1- 3 represent field mussels at 0.6, 3 and 10 km from the discharge outlet respectively, group 4 represents the wild source population. No statistical difference between the groups. (mean ± standard error (box), n=15).

Fig. 7. Volume of basophilic cells in mussel digestive gland tissue from the groups indicated. Groups 1- 3 represent field mussels at 0.6, 3 and 10 km from the discharge outlet respectively, group 4 represents the wild source population. Mean, standard error (box) and standard deviation (outer line). Groups labelled with the same letter are not significantly different from each other (ANOVA, Tukey $p<0.05$, n=5).

Fig. 8. Neutral lipid accumulation in the digestive gland tissue of mussels from the groups indicated. Groups 1- 3 represent field mussels at 0.6, 3 and 10 km from the discharge outlet respectively, group 4 represents the wild source population. Mean, standard error (box) and standard deviation (outer line). Groups labelled with the same letter are not significantly different from each other (ANOVA, Tukey $p<0.05$, n=10).

Fig. 9. Star plots showing the integrated biological response (IBR/n) in mussels from the three transplanted groups (1-3) following 6 weeks exposure. Group 4 represents mussels from the source population. IBR/n calculated from the area generated by the 7 normalised biomarker responses: CI, condition index; MN, micronuclei; NRR, Neutral red retention time; NL, Neutral lipid; VvBAS, volume of basophilic cells; CEA, cellular energy allocation; SS, Stress on stress.

Table 1. The available energy (E_a) in the digestive gland of mussels calculated from the different fractions: lipid, protein and carbohydrate. The Ea divided by the energy consumed (E_c) was used to calculate the cellular energy allocation (CEA) (mean ± SE, n=15). No statistical significant differences between the groups.

Mussel group	Carbohydrate (mJ/ mg w.w.)		Protein (mJ/mg w.w.)		Lipid (mJ/mg w.w.)	
	Mean	SD	Mean	SD	Mean	SD
1	732	239	4350	618	890	166
2	602	196	4476	708	912	186
3	575	214	4404	777	922	172
4	528	188	4187	429	930	180
	E_a (mJ/mg w.w)		E_c (mJ/mg/h)		CEA	
	Mean	SD	Mean	SD	Mean	SD
1	5984	778	14	6	530	266
2	5991	622	14	5	507	229
3	5900	693	13	5	506	190
4	5645	485	13	6	545	295

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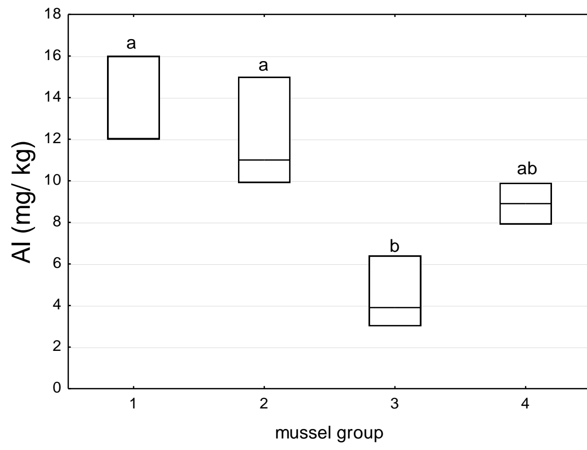
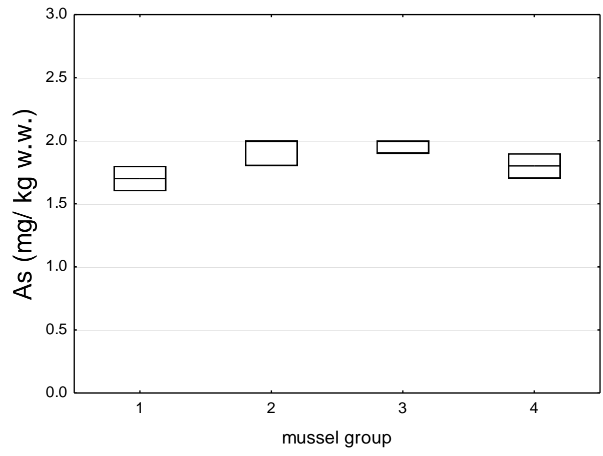
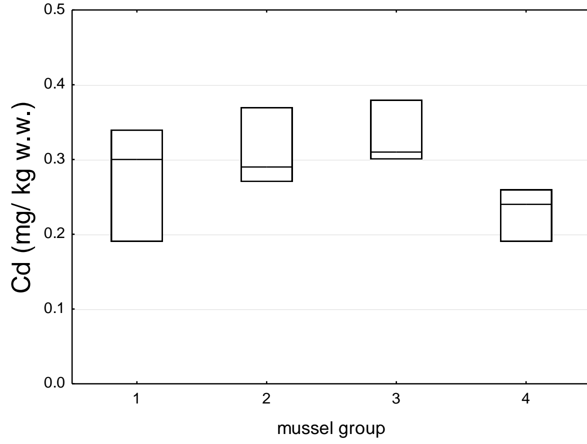
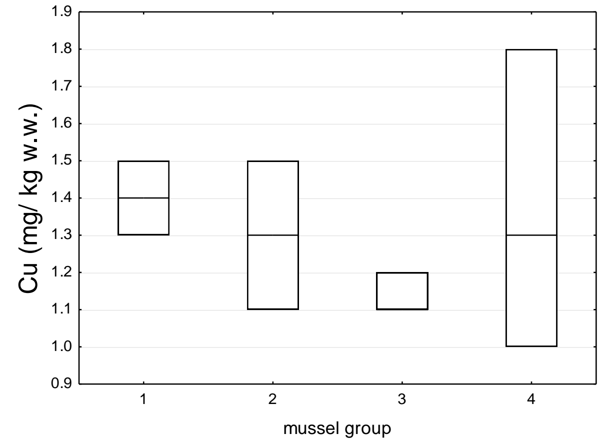
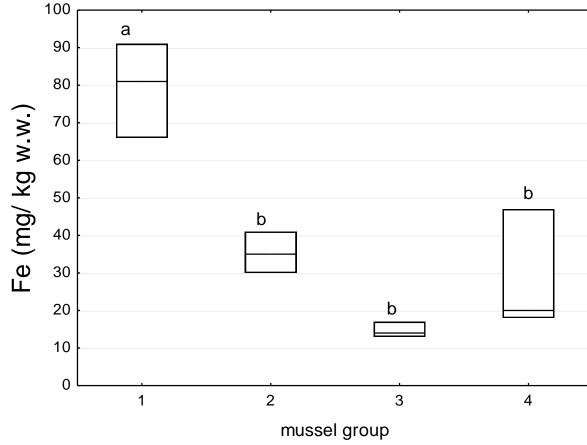
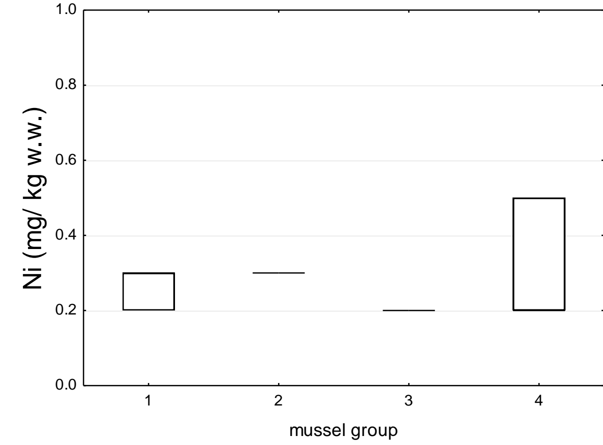

Al (mg/ kg)
mussel group
a
a
b
ab
0
2
4
6
8
10
12
14
16
18
1
2
3
4

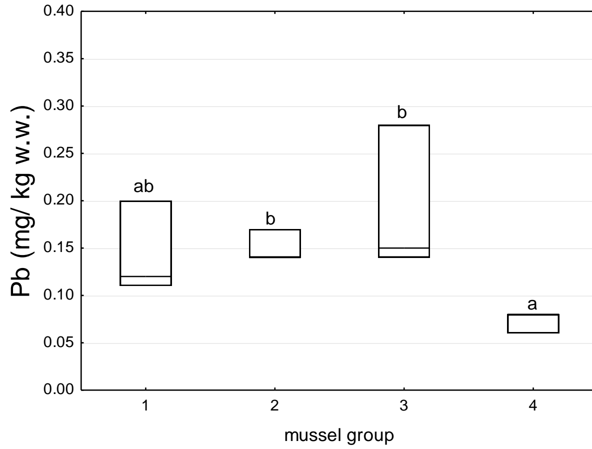

As (mg/ kg w.w.)
mussel group
0.0
0.5
1.0
1.5
2.0
2.5
3.0
1
2
3
4


Cd (mg/ kg w.w.)
mussel group
0.0
0.1
0.2
0.3
0.4
0.5
1
2
3
4


Cu (mg/ kg w.w.)
mussel group
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
1
2
3
4


Fe (mg/ kg w.w.)
mussel group
a
b
b
b
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4


Ni (mg/ kg w.w.)
mussel group
0.0
0.2
0.4
0.6
0.8
1.0
1
2
3
4


Pb (mg/ kg w.w.)
mussel group
ab
b
b
a
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
1
2
3
4

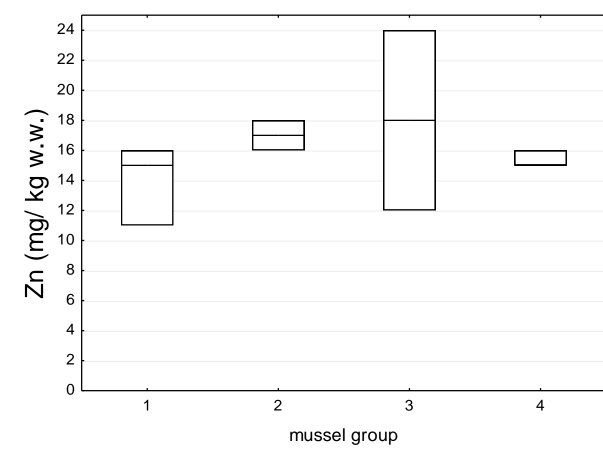
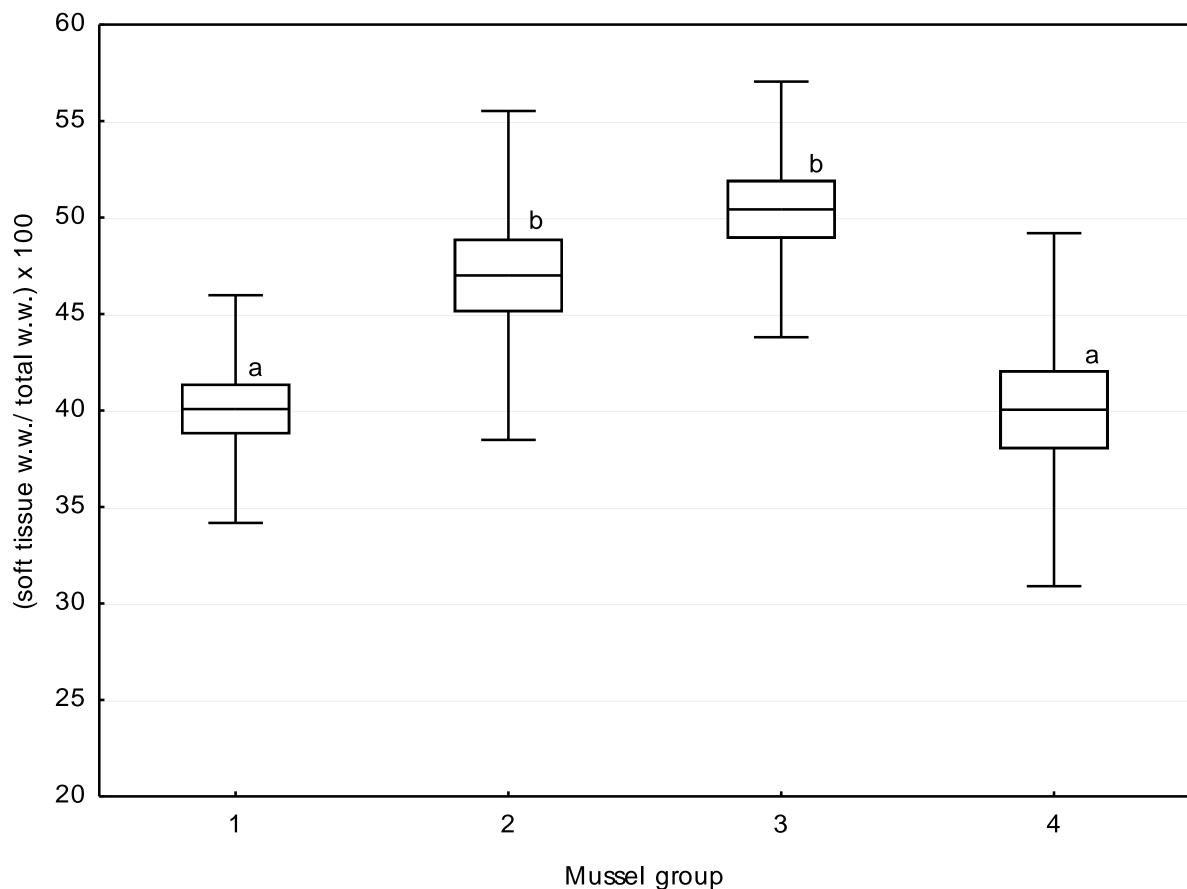

Zn (mg/ kg w.w.)
mussel group
0
2
4
6
8
10
12
14
16
18
20
22
24
1
2
3
4

Fig. 2. Metal concentrations in whole mussel homogenates. Groups 1- 3 represent field mussels, group 4 represents the wild source population (mg/ kg w.w., median ± quartiles, n=3). Values with different letters (a, b, c) are significantly different from each other ($p<0.05$). Ba and Co were below the limit of detection (LOD) of 0.2 and 0.1 mg/kg w.w. respectively and not shown.



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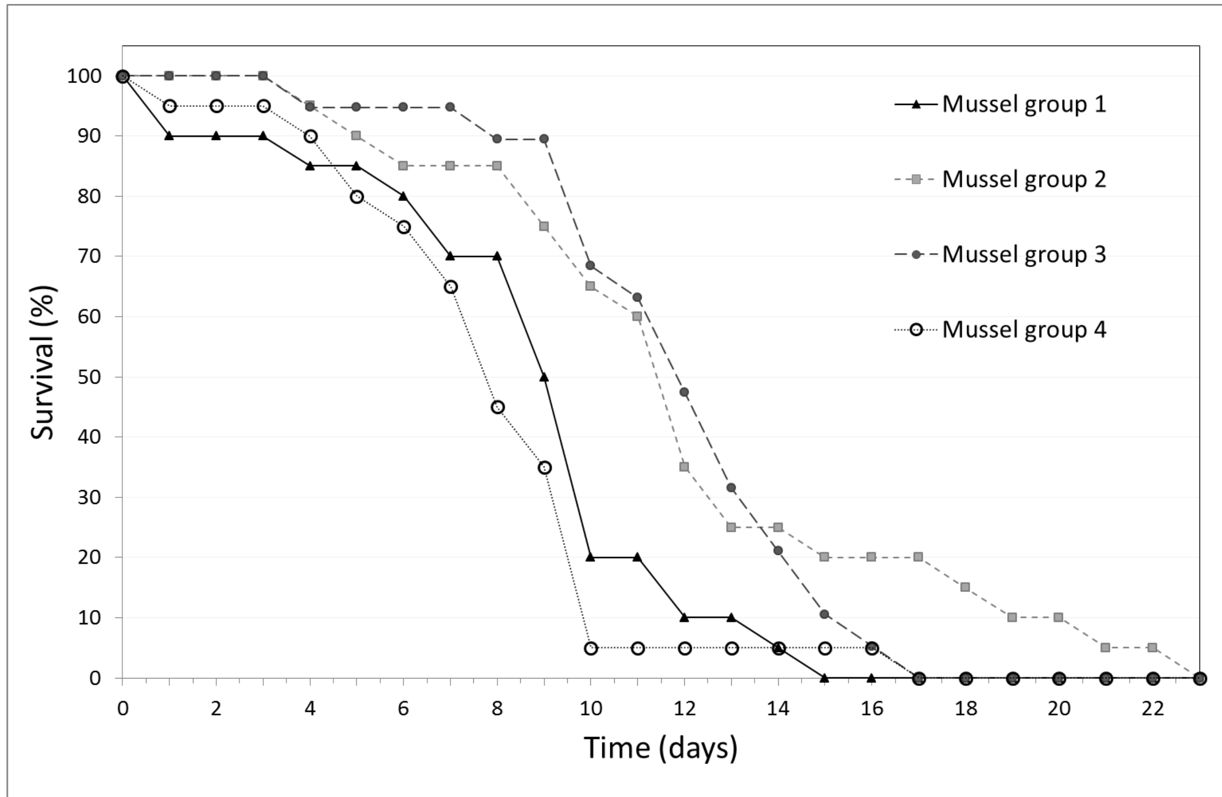

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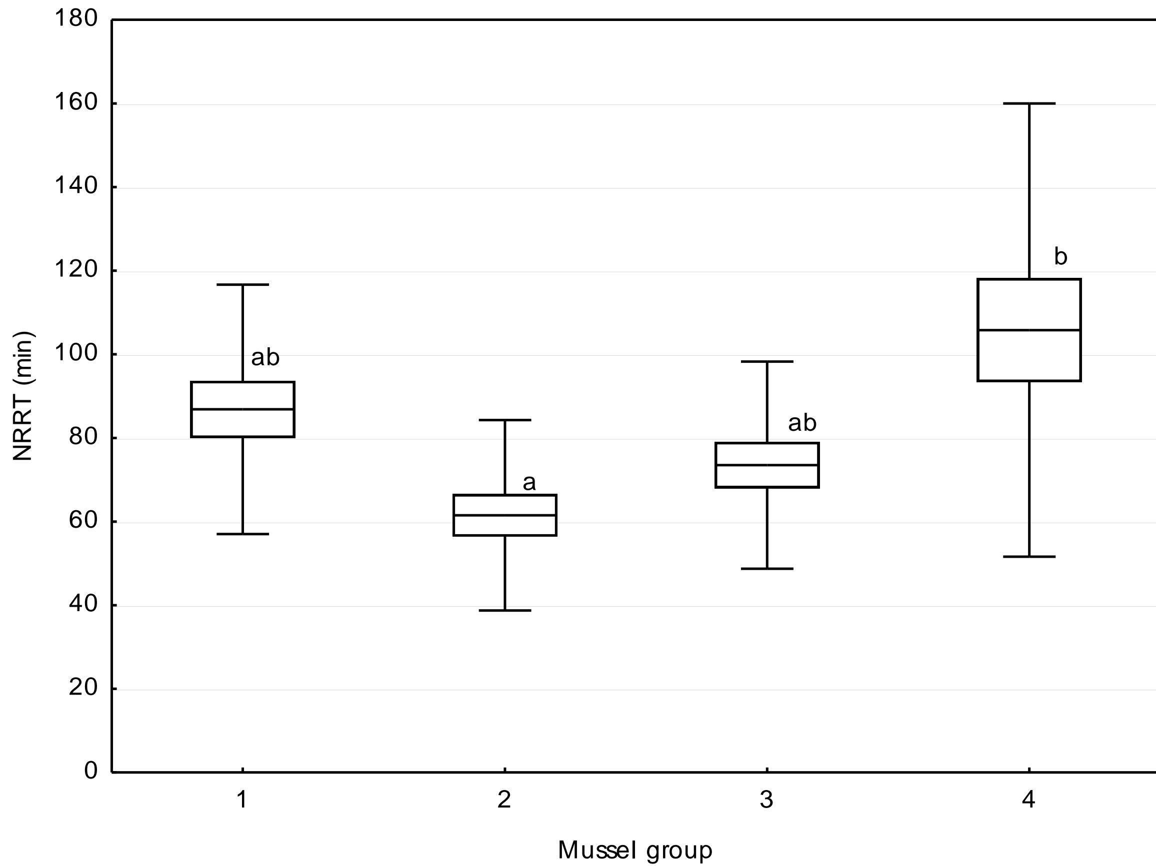

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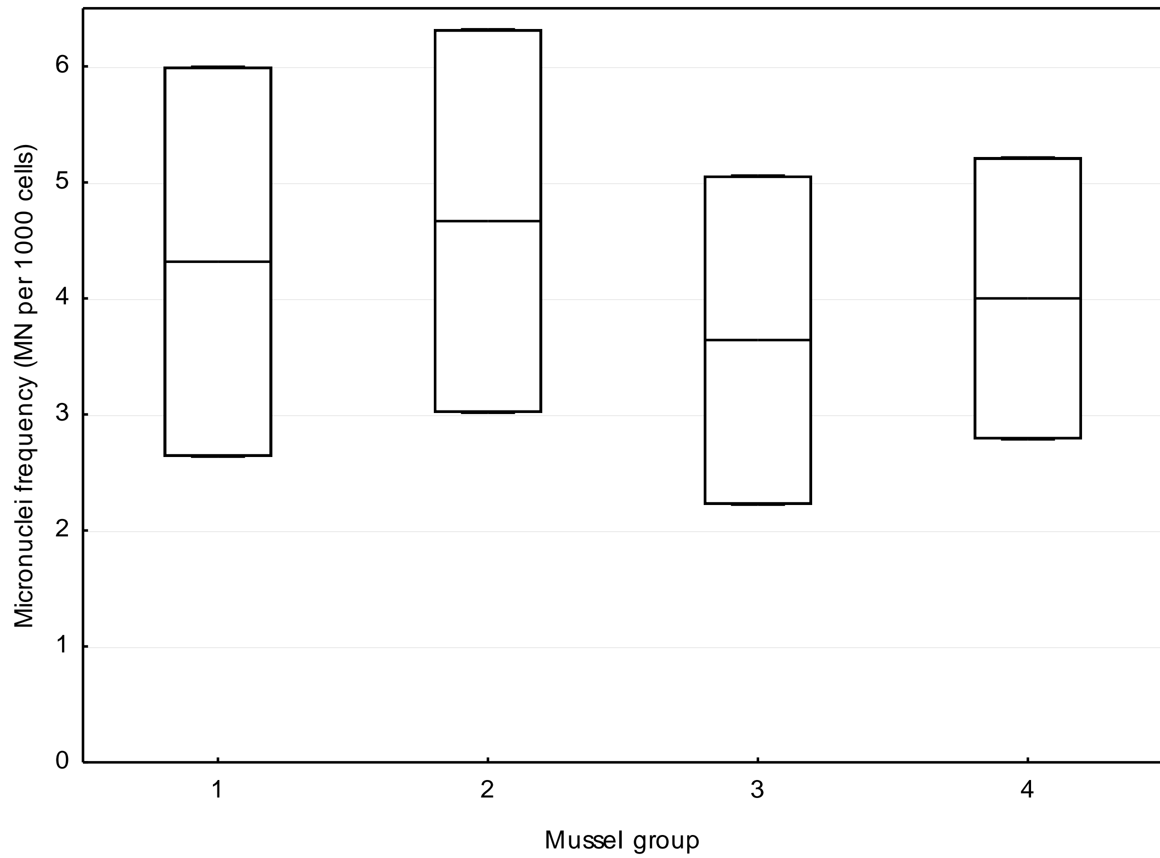

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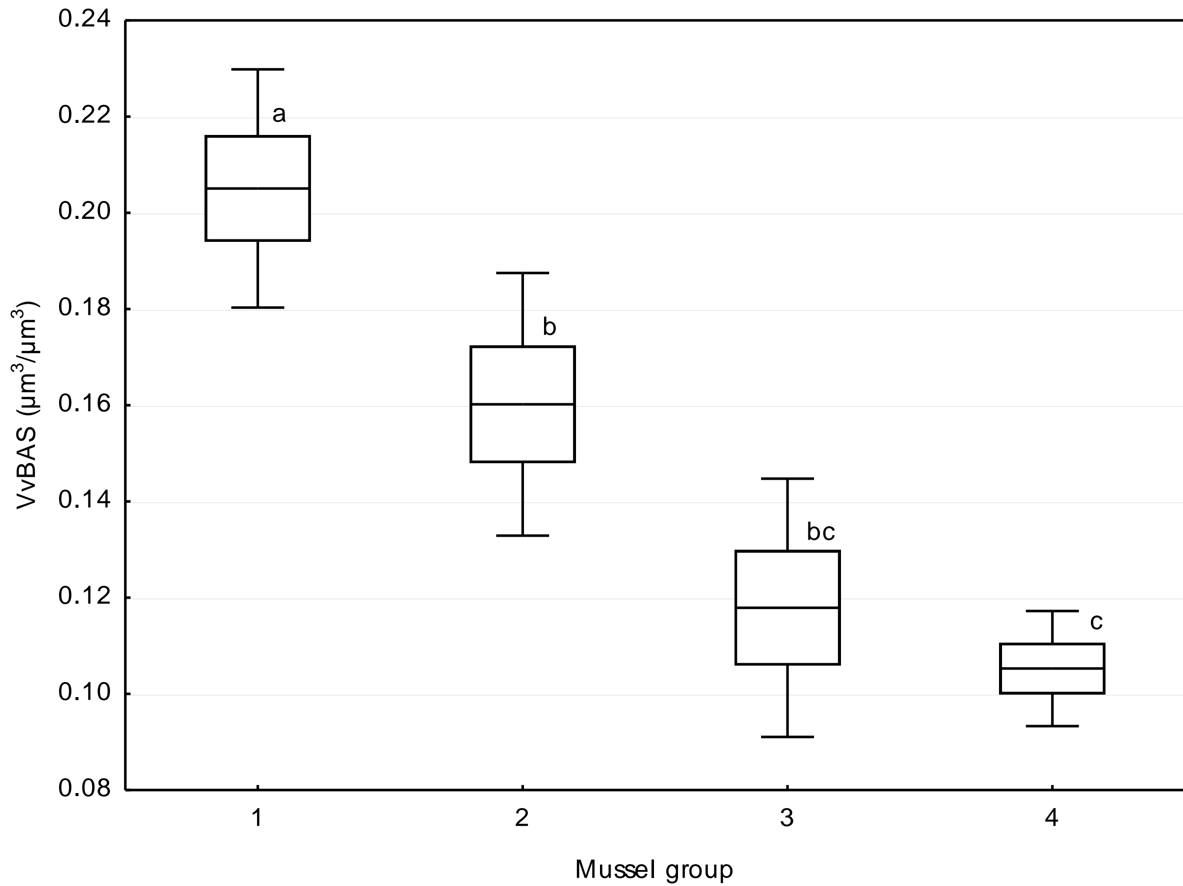

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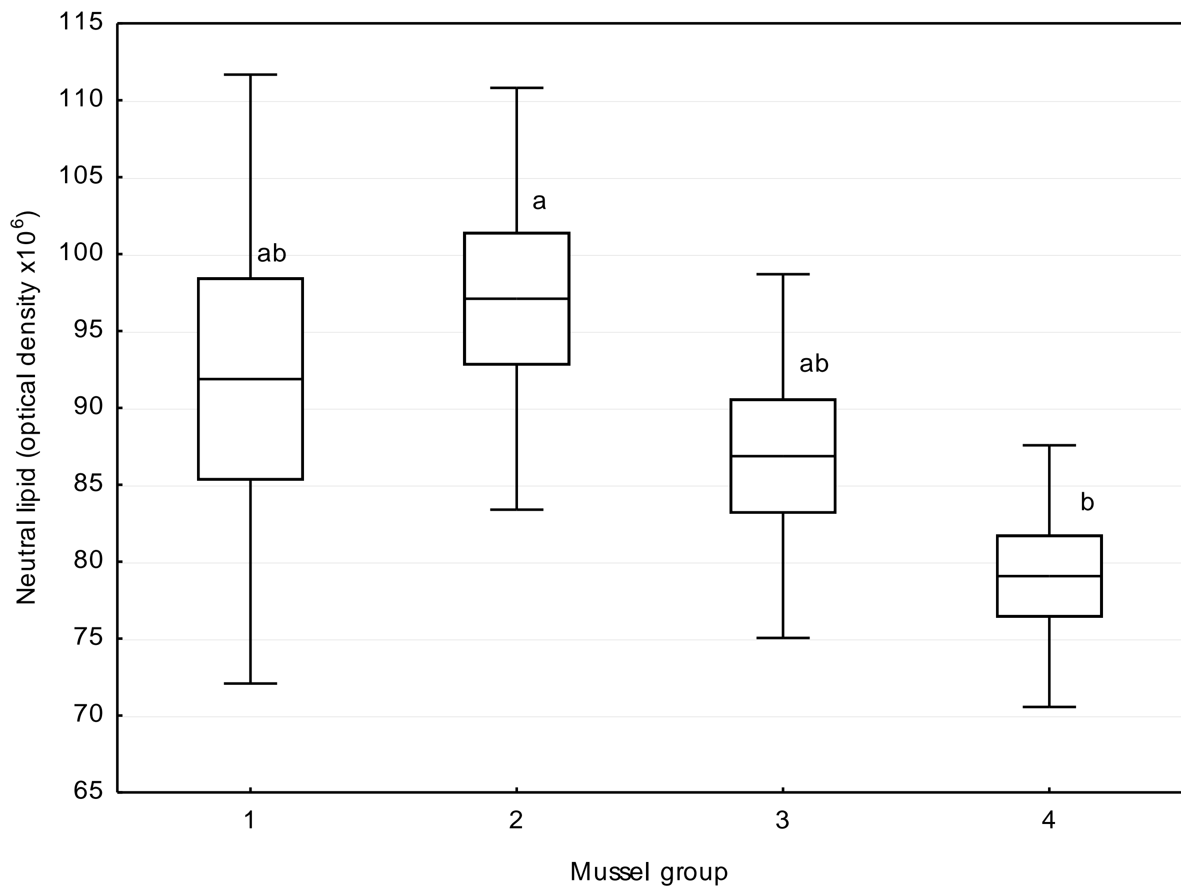
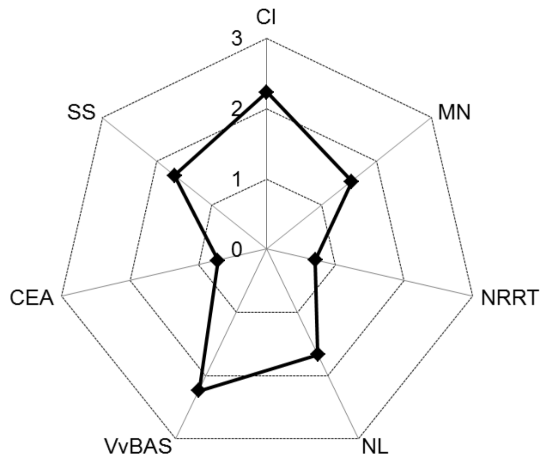
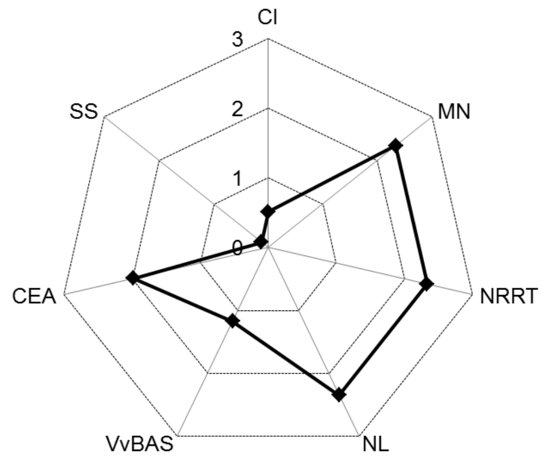
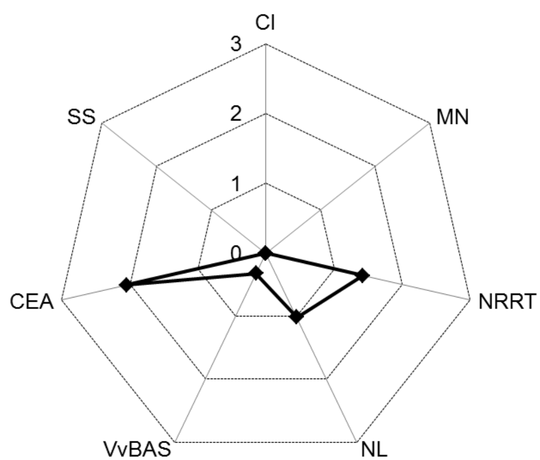
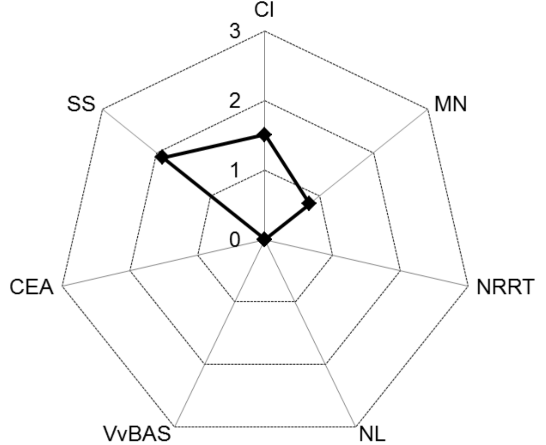


Fig. 8. Neutral lipid accumulation in the digestive gland tissue of mussels from the groups indicated. Groups 1- 3 represent field mussels at 0.6, 3 and 10 km from the discharge outlet respectively, group 4 represents the wild source population. Mean, standard error (box) and standard deviation (outer line). Groups labelled with the same letter are not significantly different from each other (ANOVA, Tukey $p<0.05$, $n=10$).









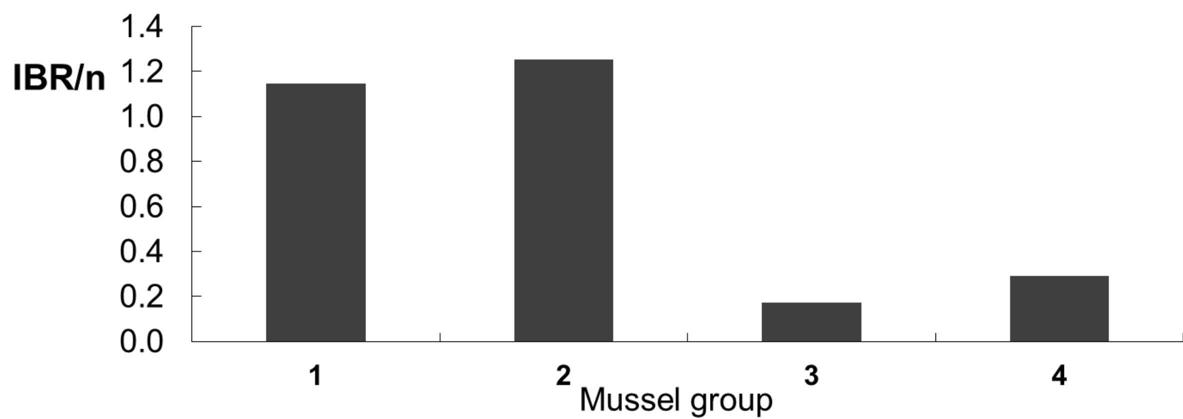


Fig. 9. Star plots showing the integrated biological response (IBR/n) in mussels from the three transplanted groups (1-3) following 6 weeks exposure. Group 4 represents mussels from the

source population. IBR/n calculated from the area generated by the 7 normalised biomarker responses: CI, condition index; MN, micronuclei; NRR, Neutral red retention time; NL, Neutral lipid; VvBAS, volume of basophilic cells; CEA, cellular energy allocation; SS, Stress on stress.