Differences in copper bioaccumulation and biological responses in three *Mytilus* species

Running title: Bioaccumulation and biomarkers in *Mytilus* spp.

Steven J Brooks*[1], Eivind Farmen[1,2], Lene Sørlie Heier[3,5], Esther Blanco-Rayón [4], Urtzi Izagirre [4],

* Corresponding author:

[1]Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, NO-0349 Oslo, Norway.

Mob: +47 92696421, Tel: +47 22185100, Fax: +47 22185200, email: sbr@niva.no

[2] Norwegian Environment Agency, Strømsveien 96, 0663 Oslo, Norway

[3] Norwegian University of Life Sciences (NMBU), Department of Environmental Sciences, P.O. Box 5003, N-1432 Aas, Norway.

[4] CBET Research Group, Research Centre of Experimental Marine Biology and Biotechnology (PiE - UPV/EHU) Basque Country, Spain.

[5] Norwegian Public Roads Administration, P.O. Box 1010, 2605 Lillehammer, Norway.

Abstract

Mytilus species are important organisms in marine systems being highly abundant and widely distributed along the coast of Europe and worldwide. They are typically used in biological effects studies and have a suite of biological effects endpoints that are frequently measured and evaluated for stress effects in laboratory experiments and field monitoring programmes. Differences in bioaccumulation and biological responses of the three *Mytilus* species following exposure to copper (Cu) were investigated. A laboratory controlled exposure study was performed with three genetically confirmed *Mytilus* species; *M. galloprovincialis*, *M. edulis* and *M. trossulus*. Chemical bioaccumulation and biomarkers were assessed in all three *Mytilus* species following a 4 day and a 21 day exposure to waterborne copper concentrations (0, 10, 100 and 500 µg/L). Differences in copper bioaccumulation were measured after both 4 and 21 days, which suggests some physiological differences between the species. Furthermore, differences in response for some of the biological effects endpoints were also found to occur following exposure. These differences were discussed in relation to either real physiological differences between the species or merely confounding factors relating to the species natural habitat and seasonal cycles. Overall the study demonstrated that differences in chemical bioaccumulation and biomarker responses between the *Mytilus* spp. occur with potential consequences for mussel exposure studies and biological effects monitoring programmes. Consequently, the study highlights the importance of identifying the correct species when using *Mytilus* in biological effects studies.

Keywords:

Mussels; biomarkers; bioaccumulation; *Mytilus edulis*; *Mytilus galloprovincialis*; *Mytilus trossulus*

1. Introduction

The *Mytilus* species have been frequently used in biological effects studies in both laboratory exposure tests and biomonitoring programmes worldwide. This is mostly due to their ability to bioaccumulate contaminants from the water column as well as having a large number of validated biological effect endpoints that can be measured and quantified. Mussels are a recommend biomonitoring organism of the International Corporation for the Exploration of the Seas (ICES) integrated biomonitoring scheme (ICES, 2011) and routinely used within national monitoring programmes (e.g. Mussel Watch (Goldberg et al., 1978); Artic Marine and Assessment Program (AMAP) (Christensen et al., 2002; Dietz et al., 2000); Prestige Oil Spill Biomonitoring (Marigómez et al. 2013); Biological Effects of Environmental Pollution on Marine Coastal Ecosystems (Lehtonen & Schiedek, 2006); Norwegian Water Column Monitoring (Brooks et al., 2011; Hylland et al., 2008), providing information on the health status of particular water bodies.

It has been frequently assumed that the mussels collected from the field and then used within biological effects studies are a distinct species whether being *Mytilus edulis* (Lmk.) in Northern Europe, *M. galloprovincialis* (Lmk.) in Southern Europe and *M. trossulus* (Gould) within the Baltic and North Atlantic coast of America. However, this generalisation has been increasingly challenged by recent studies that have shown *Mytilus* species in a patchy distribution with all three *Mytilus* spp. and hybrids occurring within the same populations (Väinölä and Strelkov, 2011; Kijewski et al., 2011; Brooks and Farmen, 2013). Since environmental factors can have significant effects on the mussel's external morphology (Seed, 1968; Akester and Martel, 2000), it is not always possible to reliably distinguish between *Mytilus* species by mere visual inspection. This can therefore lead to misrepresentation of *Mytilus* with potential implications for biomonitoring programmes and exposure studies using transplanted wild mussels as well as for aquaculture.

The ability of the different *Mytilus* spp. to bioaccumulate contaminants is not fully known, although some studies have reported differences. For example, higher concentrations of heavy metals were measured in *M. trossulus* compared to *M. edulis* in mussels collected from the same field population and in the same size category (Lobel et al., 1990). The authors suggested slower growth rates in *M. trossulus* rather than differences in metal metabolism between the two species.

Consequently, *M. trossulus* within the same size category as *M. edulis* would tend to be older and experience a longer exposure history and opportunity to bioaccumulate.

Biological responses or biomarkers can be defined as cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism and are indicative of xenobiotic exposure and/or effect (Lam and Gray, 2003). Differences in biomarkers between the *Mytilus* species have to the authors knowledge not been fully investigated. However, species differences in genotoxic response have been indicated with different background assessment criteria (BACs) for the three *Mytilus* species with respect to the frequency of micronuclei in haemocyte cells (ICES, 2011). Histological parameters including, adipogranular rate and gonadal status were found to be statistically different between *M. edulis*, *M. galloprovincialis* and their hybrids sampled from the same location in the UK (Bignell et al., 2008). Additionally, the differences in the mussel's general physiology and behaviour can have impacts on fitness and biological response. For instance, differences in reproductive strategy (i.e. spawning times) of *M. edulis* and *M. galloprovincialis* have been found to occur (Hilbish et al., 2002). Such differences in spawning are likely to impact energy budgets between the species and influence general fitness at different times of the year. Furthermore, *Mytilus* species have been found to be differentially susceptible to parasitism (Coustau et al. 1991), which may suggest some underlying difference in general physiology. Hence, there is increasing evidence to suggest that the biological responses between the *Mytilus* species do exist, although there remains a lack of controlled laboratory investigations, which are needed to measure the full extent of these potential differences.

Thus, the main aim of this study was to determine if differences in chemical bioaccumulation and biomarker responses occur between the three *Mytilus* species *M. edulis*, *M. trossulus* and *M. galloprovincialis* following a relatively short (4 days) and a longer (21 days) exposure to acute waterborne copper concentrations. The biological responses measured in mussels after 4 days included the genotoxic biomarker, micronuclei formation (Baršienė et al., 2010); and the oxidative stress biomarker glutathione (Regoli & Principato, 1995). These biomarkers are known to respond relatively quickly to contaminant stress, and in particular to copper, with 4 days copper exposure considered a sufficient amount of time to cause a measureable response. Moreover, lysosomal membrane stability

(LMS), and lysosomal structural changes (LSC), as well as neutral lipid (NL) and lipofuscin (LF) accumulation in the mussel digestive gland were measured after 21 days of exposure. The biological responses selected are known to be responsive to copper exposure and are widely used mussel biomarkers in both exposure studies and biomonitoring programmes. The findings will be discussed in relation to potential implications for biological effects studies and biomonitoring programmes.

2. Material and methods

2.1. Collection of mussels and acclimation

Mussels were collected from three separate locations, which were known to contain one of the three *Mytilus* species. *M. edulis* were collected in late autumn from the lower intertidal zone at low tide at the Outer Oslo fjord, Norway (59.488 N 10.498 E), and brought back to the NIVA marine research station in Solbergstrand, Norway. *M. trossulus* were collected in late autumn from Tingvoll fjord, west coast of Norway (62.81 N 8.275 E), and transported within an ice-cooled cooler box to the NIVA marine research station within 4 h. *M. galloprovincialis* were collected from low intertidal zone in winter at Mundaka in the Biscay Bay (43.410 N 2.698 W), and were carefully packed in an ice-cooled styrene box and transported by overnight airfreight to the NIVA marine research station. All mussels on arrival were placed in aquaria of flowing seawater, with the temperature maintained at 15 ± 2^{o}C for 4-6 weeks prior to testing. This acclimation period was considered sufficient for the mussels to adapt to the controlled laboratory conditions before testing (Widdows and Bayne, 1971; Altieri, 2006). No mortalities were recorded during transport and acclimation. The mussels were fed daily with a concentrated solution of Shellfish diet 1800® containing a mixture of four marine microalgae (Reed Mariculture Inc. USA).

2.2. Exposure to copper

The three mussel species were exposed to high, middle and low stable doses of copper chloride ($CuCl_2$) solution within a flow-through seawater system. With an established filtered (10 µm) seawater flow rate of 3 L/ min at 15 ± 2^{o}C and 33 ‰ salinity, a concentrated solution of $CuCl_2$ was dosed into the seawater inlet pipe, to provide total nominal copper concentrations of 10, 100 and 500 µg/ L (0.16,

1.57 and 7.87 µM Cu). Fifty mussels of each group were exposed per treatment. The three mussel species of each exposure condition were placed inside the same glass aquarium and separated by a perforated plastic screen that allowed water flow but prevented mixing of the mussels. The flow-through set-up was designed to ensure that all mussels within the same aquaria received identical exposure to copper. The mussels were fed every two days with of Shellfish diet 1800® and sub-sampled after 4 days and after 21 days of exposure.

2.3. Mussel sampling

After 4 days, a sub-sample of the mussels from each group was removed from the exposure tanks and sampled for haemolymph and selected tissues. The haemolymph was removed from the abductor muscle with a hypodermic syringe and used to measure micronuclei formation. The digestive gland, gills and gonad were excised and placed in separate labelled cryovials before being snap frozen in liquid nitrogen. Each mussel was given a code number that was independent of the exposure dose, which enabled the biomarker analysis to be performed blind. The digestive gland was used to measure GSH whilst the gonad was used to confirm *Mytilus* species. In addition, the whole soft tissue of mussels were excised from their shells and placed in individual 50 mL Nalgene containers. Three replicates of five mussels from each treatment and species group were excised. The samples were stored at -20^{o}C until required for copper analysis.

After 21 days the remaining mussels were sampled. Digestive gland, gonad and gill tissue were removed from individual mussels and placed in pre-coded cryovials and snap frozen in liquid nitrogen. The digestive gland was used to measure histochemistry (LMS, LSC, NL, LF), and the gonad was used to confirm *Mytilus* species. Three replicates of the whole soft tissue of five mussels were excised from their shells and placed in individual 50 mL Nalgene containers. The samples were stored at -20^{o}C until required for copper analysis.

All mussels were identified to *Mytilus* species from DNA isolated from gonad tissue samples, followed by PCR amplification and gel electrophoresis. The individual species were determined before samples were selected for bioaccumulation and biomarker measurements to ensure that only pure *M. edulis, M. trossulus* and *M. galloprovincialis* were analysed.

2.3. DNA isolation, amplification and Gel electrophoresis

Total DNA was extracted from 20-40 mg of gonad tissue from frozen mussels using QuickExtract DNA extraction solution (Epicentre, Madison, Winsconsin, USA) following the manufacturer's recommended protocol. Briefly, the tissue was homogenised in 0.1 mL QuickExtract DNA extraction solution by vortexing for 15 seconds, incubated at 65°C for 10 min, vortexed for another 15 seconds, and finally incubated at 98°C for 2 min. The homogenates were then diluted 1:10 in molecular grade H_2O. For species identification, polymerase chain reaction (PCR) were used to amplify a specific 180 base pair (bp) segment for *M. edulis*, 168 bp segment for *M. trossulus* or 126 bp segment for *M. galloprovincialis* of the *Glu* gene (polyphenolic adhesive protein) as described by Inoue et al. (1995). The 25 µL PCR reactions contained 5 µL of DNA template, 300 µM forward and reverse primers, 2x SsoFast EvaGreen Mastermix (BioRad, Hercules, CA, USA), and were subjected to a 5 min pre-heating stage at 95ºC followed by 35 cycles of 30 sec at 95ºC, 30 sec at 55ºC, 30 sec at 72ºC, and final extension step of 10 min at 72ºC.

One µL of the PCR product was loaded onto a DNA 1000 chip (Agilent technologies, Santa Clara, California, USA) and run in a Bioanalyser instrument (Agilent technologies, Santa Clara, California, USA) for visualisation of amplicon size.

2.4. Bioaccumulation of copper

Individual whole mussel samples were selected based on the results of the mussel speciation assessment, with three mussels measured per group. Samples were defrosted, homogenised and a sub sample taken of approximately 5 g. Total copper concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP OES).

2.5. Condition index

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

2.6. Micronuclei formation in mussel haemocytes (MN)

Approximately 0.1 mL of haemolymph was removed from the posterior adductor muscle of each mussel with a hypodermic syringe containing 0.1 mL PBS buffer (100 mM PBS, 10 mM EDTA). The haemolymph and PBS buffer were mixed briefly in the syringe before being 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 brought back to the laboratory for further processing. 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 micronuclei formation was measured on coded slides without knowledge of the exposure status of the samples to eliminate bias. The frequency of micronuclei in haemocytes was determined microscopically at 100x objective (final magnification ~1000x). A total of 2000 cells were examined for each experimental group of mussels. Only cells with intact cellular and nuclear membranes were scored. MN were scored when: (a) nucleus and MN have a common cytoplasm; (b) colour intensity and texture of MN is similar to the nucleus; (c) the size of the MN is equal or smaller than 1/3 of the nucleus; and (d) MN are apparent as spherical structures with a sharp contour (Bolognesi and Fenech, 2012).

2.7. Reduced glutathione

Reduced glutathione (GSH) was measured in cytosolic fraction of digestive gland samples by conjugation of monochlorobimane (mCB) (Life Technologies Ltd, Paisley, UK) to the sample GSH, forming a stable and fluorescent product (Kamenic et al., 2000). All samples were diluted 1:10 to obtain protein concentration between 0.3 and 1 mg/mL and a GSH standard curve was prepared with dilutions ranging from 1.5 to 100 µM. Standards and samples were pipetted into 96-well microtiter plates in three and four replicates of 50 µL respectively. Finally, 50 µL of the reaction buffer, consisting of Trisbuffer pH 7.8 with 200 µM mCB and 2U/mL equine GST (Sigma Aldrich) was added to all wells. The plates were incubated at room temperature in the dark for 18 hours before

analysis in a Victor Wallac microplate reader (Perkin Elmer) at emission 486 nm and excitation 405 nm. In order to correct for quenching of fluorescence, all samples were then spiked with GSH-mCB product prepared from 1 nmole of GSH and analysed again at the same settings. Concentration of reduced GSH was determined by relating measured FU to the GSH standard curve. A correction factor for quenching was derived by calculating the difference between the expected spike FU and the spike FU actually observed in the spiked samples. Finally GSH was related to the protein concentration of each sample, to give the GSH activity in nmoles/min/mg protein (Žegura et al., 2006).

2.8 Histochemistry

2.8.1. Lysosomal membrane stability (LMS)

The determination of lysosomal membrane stability (LMS) was based on the time of acid labilisation treatment required to produce the maximum staining intensity according to UNEP/RAMOGE (1999), after demonstration of hexosaminidase (Hex) activity in digestive cell lysosomes. Eight serial cryotome sections (10 µm) were subjected to acid labilisation in intervals of 0, 3, 5, 10, 15, 20, 30 and 40 min in 0.1 M citrate buffer (pH 4.5 containing 2.5% NaCl) in a shaking water bath at 37°C, in order to find out the range of pre-treatment time needed to completely labilise the lysosomal membrane, denoted as the labilisation period (LP; min). Following this treatment, sections were transferred to the substrate incubation medium for the demonstration of Hex activity. The incubation medium consisted of 20 mg naphthol AS-BI-Nacetyl-b-D glucosaminide (Sigma, N 4006) dissolved in 2.5 mL 2-methoxyethanol (Merck, 859), and made up to 50 mL with 0.1 M citrate buffer (pH 4.5) containing 2.5% NaCl and 3.5 g low viscosity polypeptide (Sigma, P5115) to act as a section stabiliser. Sections were incubated in this medium for 20 min at 37°C, rinsed in a saline solution (3.0 % NaCl) at 37°C for 2 min and then transferred to 0.1 M phosphate buffer (pH 7.4) containing 1 mg/mL diazonium dye Fast Violet B salt (Sigma, F1631), at RT for 10 min. Slides were then rapidly rinsed in running tap water for 5 min, fixed for 10 min in Baker's formol calcium containing 2.5% NaCl at 4°C and rinsed in distilled water. Finally, slides were mounted in Kaiser's glycerine gelatin. The time of acid labilisation treatment required to produce the maximum staining intensity was assessed under the light microscope as the maximal accumulation of reaction product associated with lysosomes

(UNEP/RAMOGE 1999). Although two maximum staining peaks may be observed in some cases, the first one was always considered. Four determinations were made for each animal by dividing each section in the acid labilisation sequence into 4 approximately equal segments and assessing the LP in each of the corresponding set of segments. The mean LP value was then derived for each section, corresponding to an individual digestive gland.

2.8.2. Lysosomal structural changes (LSC)

The histochemical reaction for β-Gus was demonstrated as in Moore (1976) with the modifications described by Cajaraville et al. (1989). Frozen tissue sections (8 μm) from 10 mussels were put at 4°C for 30 min and then brought to room temperature before staining. Slides were incubated in freshly prepared β-Gus substrate incubation medium consisting of 28 mg naphthol AS-BI-β-glucuronide (Sigma, N1875) dissolved in 1.2 mL of 50 mM sodium bicarbonate, made up to 100 mL with 0.1 M acetate buffer (pH 4.5 containing 2.5% NaCl) and 15% of polyvinyl alcohol at 37 °C for 40 min in a shaking water bath and then transferred to a post-coupling medium containing 0.1 g Fast Garnet (GBC) (Sigma, F8761) dissolved in 100 mL of 0.1 M phosphate buffer (pH 7.4 containing 2.5% NaCl) in the darkness and at room temperature for 10 min. Afterwards, sections were fixed in Baker's formol calcium containing 2.5% NaCl at 4°C for 10 min and rinsed briefly in distilled water. Finally, sections were counterstained with 0.1% Fast Green FCF (Sigma, F7252) for 2 min, rinsed several times in distilled water and mounted in Kaiser's glycerol gelatine.

Five measurements using a 100x objective lens were made in each individual using image analysis (BMS, Sevisan). The mean value of the stereological parameters was determined for each mussel digestive gland (Lowe et al., 1981): lysosomal volume density (VvL), lysosomal surface-to-volume ratio (S/VL) and lysosomal numerical density (NvL).

2.8.3. Intracellular accumulation of neutral lipids (NLs)

Intracellular NLs were demonstrated histochemically by staining with Oil Red O (ORO) (Culling, 1974). Cryostat sections (8 μm) were transferred to a cabinet at 4 °C and fixed in Baker’s solution (2.5% NaCl) for 15 min. Then sections were dried at room temperature, washed in isopropanol (60%)

and rinsed in (ORO) staining solution for 20 min. The ORO stock solution is a saturated (0.3%) solution of ORO (BDH, 34061) in isopropanol. The staining solution was freshly made by dissolving 60 ml stock solution in 40 ml distilled water and filtering after a 10 min gap to stabilize the solution. The staining solution is only stable for 1-2 h. Stained sections were differentiated in 60% isopropanol, washed in water, counterstained with 1% Fast Green FCF (Sigma, F7252) for 20 min and mounted in Kaiser´s glycerine gelatine. Five measurements using a 40x objective lens were made in each individual using image analysis (BMS, Sevisan). The mean volume density of neutral lipids in digestive alveolies (Vv) was determined for each mussel.

2.8.4. Lipofuscin (LF) determination

LF content of tertiary lysosomes was quantified using Schmorl reaction (Pearse, 1972). Cryostat sections (8 μm thick) were fixed for 15 min in Baker buffer at 4°C. Then, they were rinsed in distilled water and placed in Schmorl's solution, which contains 1% ferric chloride and 1% potassium ferricyanide, at a ratio of 3: 1. Sections were stained in this solution for 5 min. After that, they were washed with 1% acetic acid for 1 min, followed by rinsing in distilled water. The slides were mounted in Kaiser´s glycerine gelatine.

2.9. Statistics

SPSS v 17.0 software (SPSS INC., Chicago, Illinois) and Statistica v11 software (Statsoft Inc), were employed for the statistical analyses. Homogeneity of variance (Levene's test) and normality of data (Kolmogorov-Smirnov's) were tested before statistical analyses (Sokal & Rohlf, 1995). Statistically significant differences among species were tested according to the Duncan's post-hoc test based on one-way analysis of variances (1-way ANOVA) for parametric variables (Cu bioaccumulation, MN, GSH, Vv, SvL, S/VL, Nv), and the Mann-Whitney's U-test for non-parametric variable (LP). Furthermore, statistically significant differences between unexposed and exposed mussels for each species were tested according to the Student t-test for parametric variables (Vv, SvL, S/VL, Nv) and the Mann-Whitney's U- test for non-parametric variable (LP) A 95% significance level ($P<0.05$) was established for all statistical analyses carried out.

3. Results

3.1. *Mytilus* species determination

The method of DNA amplification and gel electrophoresis of mussel gonad tissue was successful in differentiating between the three *Mytilus* species. Single bands were identified at 180 bp for *M. edulis* 168 bp for *M. trossulus* and 126 bp for *M. galloprovincialis*. The proportion of the *Mytilus* spp. and hybrids from the different populations are shown in table 1. As expected *M. galloprovincialis*, *M. edulis* and *M. trossulus* were the dominant mussel species in the populations collected from Bilbao, the outer Oslo fjord and Tingvoll fjord in Norway respectively. However, pure populations were not found at any of the sampling locations, with a single *M. trossulus* identified from the Bilbao population as well as five *M. galloprovincialis*/ *M. edulis* hybrids. Hybrids of *M. galloprovincialis*/ *M. edulis* (n=1) and *M. edulis/ M. trossulus* (n=3) were found in the outer Oslo fjord population. Highest variability was seen in the Tingvoll fjord population with hybrids of *M. edulis/ M. trossulus* (n=13) and *M. galloprovincialis*/ *M. trossulus* (n=3) as well as *M. edulis* found in addition to the dominant *M. trossulus*.

3.2. Copper Bioaccumulation

Copper concentrations in whole body burden of individual mussels following exposure to a range of copper concentrations after 4 and 21 days are shown in figure 1. No differences in the bioaccumulation of copper were observed between the *Mytilus* spp. after 4 days exposure to 10 and 100 µg/L Cu. However, for the highest copper concentration (500 µg/ L), Cu bioaccumulation in *M. trossulus* was markedly higher and approximately 3 times the Cu bioaccumulation of both *M. edulis* and *M. galloprovincialis*.

Both 100 and 500 µg/ L Cu were lethal to all three *Mytilus* spp. with 100% mortality recorded in the exposed mussels after 21 days exposure. Consequently, only the copper concentrations for the *Mytilus* spp. from the control and lowest copper concentration (10 µg/ L) are presented. Following the 21 day exposure to 10 µg/ L Cu, tissue copper concentrations in *M. edulis* and *M. trossulus* were markedly

higher and approximately 3 times the concentration of copper accumulated in the tissue of *M. galloprovincialis*.

The bioaccumulation of Cu in the control group for all three *Mytilus* spp. was almost identical between 1 and 2 mg/ kg after 4 and 21 days. For the 10 µg/ L exposure group, the bioaccumulation of Cu in *M. galloprovincialis* after 4 and 21 days was almost identical at approximately 6 mg/ kg. In contrast, the bioaccumulation of Cu in *M. edulis* and *M.* trossulus after 21 days (~17-18 mg / kg) was approximately double the concentration measured after 4 days (7-8 mg / kg).

3.3. Biological effects measurements following a 4 day exposure

3.3.1. Condition Index

The condition index (CI) was found to differ between the *Mytilus* spp. (Figure 2). Overall, *M. galloprovincialis* had the lower CI followed by *M. edulis* and *M.* t*rossulus*. In contrast, the CI of *M. edulis* and *M. trossulus* was lower in mussels exposed to 10 µg /L Cu for 4 days compared to their respective controls, although exposure to high copper concentrations (up to 500 µg/ L) did not decrease the CI any further to that observed at 10 µg/ L Cu.

3.3.2. Micronuclei formation

The frequency of micronuclei in the haemocytes of the *Mytilus* spp. following 4 day Cu exposure is shown in figure 3. In the control groups, the micronuclei frequency was markedly higher in *M. edulis* compared to the other two. Following 4 day Cu exposure, *M. edulis* appeared to be the most sensitive with a significant increase in micronuclei at 10 µg/ L. In comparison, micronuclei frequency was significantly increased in *M. galloprovincialis* at 100 µg/ L. In *M. trossulus*, 500 µg/ L was required to increase micronuclei frequency from control levels, although the level of significance was marginally above $p<0.05$ at 0.052.

3.3.4. Reduced glutathione (GSH)

Reduced glutathione (GSH) concentrations in the digestive gland of the mussels showed very little difference between the *Mytilus* species or between nominal Cu exposures with no significant differences recorded (Figure 4). Mean GSH concentrations in all groups were between 4.5 and 9 nmol/ mg protein.

3.4. Biological effects measurements following a 21 day exposure

3.4.1. Condition Index

As previously observed for CI after 4 days exposure, *M. galloprovincialis* had a lower CI than *M. edulis* and *M. trossulus* (Figure 5). The CI was unresponsive to copper exposure after 21 days in all three mussel species.

3.4.2. Histochemistry

Lysosomal membrane stability (LMS)

No significant differences were found in LMS between control *Mytilus spp*., neither between exposed *Mytilus spp*. (Figure 6). In the comparison between control and exposed groups of each species, only *M. trossulus* showed significant decrease in LMS after 21 days.

Lysosomal structural changes (LSC)

Differences between species were recorded for lysosomal volume density (Vv) and lysosomal surface to volume ratio (S/V) (Figure 7). In the control groups, Vv was significantly lower in *M. galloprovincialis* compared to *M. edulis* and *M. trossulus* (Figure 7A). Moreover, higher S/V values were recorded in *M. galloprovincialis* than in *M. edulis and M. trossulus*. In exposed groups, *M. galloprovincialis* showed significantly lower Vv and higher S/V than *M. edulis* (Figure 7B). In addition, significant decreases on Vv values were observed in *M. edulis* and *M. trossulus* after Cu exposure to 10 µg/ L for 21 days. Lysosomal numerical density (Nv) showed no significant differences neither between species, nor among control and exposed mussels (Figure 7C).

Intracellular accumulation of neutral lipids

Intracellular neutral lipid (NL) accumulation of unexposed *M. edulis* was significantly higher than unexposed *M. galloprovincialis* (Figure 8). However, in *M. edulis*, NL accumulation tended to decrease after 10 µg Cu/ L exposure for 21 days. Regarding *M. trossulus*, their Vv values in control and exposed groups were between those of the other two species. Although following Cu exposure NL accumulation in *M. trossulus* tended to increase.

Lipofuscin determination

Differences in the content of lipofuscins were observed between species and, as well as, between controls and exposed mussels (Figure 9). *M. galloprovincialis* showed higher concentration of lipofuscins than *M. edulis* and *M. trossulus*. In all mussel species less lipofuscin content was observed after 10 µg Cu/ L exposure for 21 days. In addition, *M. edulis* appeared to have smaller lipofuscins than the other two species.

4. Discussion

The patchy distribution of *Mytilus* species around the European coast and elsewhere has made it difficult to assume the correct species without first confirming through genetic identification (Brooks & Farmen, 2013; Kijewski et al. 2011). For instance, of the 85 mussels analysed from the Northern coast of the Basque Country, which were initially considered to be entirely *M. galloprovincialis*, 5 individuals of *M. galloprovincialis*/ *edulis* hybrids as well as a single *M. trossulus* individual was detected. Likewise, *Mytilus* populations from the Oslo fjord had 95% of pure *M. edulis* and 5% of hybrids. In contrast, the Tingvoll fjord nr Molde, Norway had a mixed population despite having a dominate species present. Therefore, genetic species identification should be used if specific *Mytilus* species are required, which may be of significance when for example the bioaccumulation and biomarker responses to chemical stress differ between the species.

Although it has been relatively well documented that metal bioaccumulation in mussels is variable with certain metals (Rainbow, 2007), differential uptake of one metal (i.e. Cu) between mussel species is less well defined. Marked differences in copper bioaccumulation were clearly evident between the *Mytilus* species following both a 4 day and a 21 day exposure to elevated copper concentrations. To the author's knowledge, the current study was the first time a comparative laboratory exposure has been performed on the three *Mytilus* species. Previous evidence of differences in metal bioaccumulation between *Mytilus* species was reported in mussels collected from field populations where *M. edulis* and *M. trossulus* co-existed (Lobel et al., 1990). The increased metal bioaccumulation in *M. trossulus* was explained by the authors in terms of the slower growth rate of this species compared to *M. edulis*. Consequently, mussels of the same size range represented older *M. trossulus* than *M. edulis* and therefore a longer exposure history. However, this explanation would not explain the observations within a controlled laboratory experiment, where the exposure duration and concentrations were known. The significantly higher concentrations of copper found in whole soft tissue homogenates of *M. trossulus* compared to *M. edulis* and *M. galloprovincialis* after 4 days are therefore more likely to represent a physiological difference between the species. Possible reasons for the observed differences in metal bioaccumulation may include either the varying ability to sequester and/ or excrete metals from *Mytilus* tissue (Ackerman & Nishizaki, 2004) as well as possible

differences in the cellular lipid content between the species. However, alternatively it is not possible to completely eliminate confounding factors that may have arisen due to the mussels being collected from different habitats. For example, differences in exposure histories, seasonal reproductive cycles, and physicochemical differences between their natural environmental conditions. Also the seasonal temperature in which the mussels had been collected in their respective sampling sites and the temperature used along the experiment were different for the different species (http://www.seatemperature.org). Procedures were taken to reduce these external factors as far as possible, such as collecting mussels from similar environments of low physical and contaminant stress and a long acclimation period in the laboratory prior to testing, however such factors cannot be fully excluded.

After the three week exposure only the mussels from the 10 µg/ L Cu exposure group and the control group were available due to total mortalities at 100 µg/L and 500 µg/L Cu, although incidentally there was no apparent difference in the acute toxicity of copper to the different *Mytilus* species. Differences in copper bioaccumulation did occur after 21 days exposure, although somewhat different to that observed after only 4 days. Bioaccumulation was almost identical between *M. edulis* and *M. trossulus* after 21 days, which were both significantly higher than copper measured in *M. galloprovincialis*. Whether differences in metal body burden remain after longer exposure durations of months or years may have more implications to aquaculture practices. However, the differences observed after 4 and 21 days indicate that some physiological variation exists, which could influence the outcome of laboratory exposures and/or field transplantation studies using mixed populations.

Further differences in metal bioaccumulation in mussels have been reported in field populations, with differences attributed to tidal height, body size and condition index (Mubiana et al., 2006). In the present study, condition index was found to differ between the species, with overall a higher condition index observed in the order *M. trossulus*, *M. edulis* and *M. galloprovincialis.* Since this appears to pattern metal bioaccumulation between the species, with a significant positive correlation between condition index and copper bioaccumulation ($p<0.05$, Spearman's rank coefficient), it may provide some insight into the observed differences. Since the *Mytilus* individuals were all collected from different locations (i.e. Basque Country, Northern Norwegian coast and outer Oslo fjord, Norway),

difference in their seasonal cycles and overall reproductive condition are likely to vary. For instance, *M. galloprovincialis* experience a seasonal temperature range of 12°C in March up to 21°C in August. In contrast, the Norwegian mussels, *M. edulis* and *M. trossulus* have a seasonal temperature range of 2°C to 14°C and 5°C to 17°C in March and August respectively. The mussels were collected between October and November where average water temperatures were 9, 9.9 and 16.5°C for *M. edulis*, *M. trossulus* and *M. galloprovincialis* respectively (www.seatemperature.org). The exposure was performed at 15°C and differences in temperature acclimation and effects on body metabolism and physiological processes may be a contributing factor for the observed differences in bioaccumulation.

4.1. Biomarker responses

Micronuclei formation is widely used as a sensitive measure of genotoxicity in mussels (Barsiene et al. 2010; Brooks et al., 2011). ICES assessment criteria exist for *Mytilus* species, with separate assessment criteria suggested for *M. edulis*, *M. trossulus* and *M. galloprovincialis* of 2.5, 4.5 and 3.9 micronuclei per 1000 nuclei respectively (ICES, 2011; Davies and Vethaak, 2012). These assessment criteria have been established from data available in mussels from European coastal waters as opposed to comparative laboratory studies (Davies and Vethaak, 2012). However, they do recognise that differences in response occur when exposed to contaminant stress. In our laboratory exposure, *M. edulis* individuals were found to be the most sensitive of the mussels with respect to micronuclei formation, with an increase in micronuclei frequency following four days exposure to the lowest nominal copper concentration. The lower assessment criteria for this species, appears to support the view that it is the most sensitive genotoxic response of the three *Mytilus* species. Additionally, the micronuclei response of *M. galloprovincialis* at 100 µg/L and *M. trossulus* at 500 µg/L after 4 days also corresponds with the rank order of the assessment criteria with the latter being the least sensitive.

Reduced glutathione (GSH) plays a central role in cellular antioxidant defence as well as being involved in metal sequestration and detoxification. The lack of response of glutathione in the digestive gland of all *Mytilus* species, despite exposure to high copper concentrations, would suggest that 4 days exposure was not suitable for the optimal up regulation of these proteins in the mussel tissues. However, there is evidence that shows a reduction in GSH in mussels following only one day

exposure to copper (Canesi et al., 1999). In contrast, Regoli & Principato (1995), found elevated levels of GSH in *Mytilus* digestive gland following a 3 week exposure to copper. In the present study, elevated copper concentrations in the mussel tissues were evident after only 4 days, indicating a clear exposure of the mussel to the metal. The lack of significant response does appear to be related to the exposure duration, although whether the exposure duration should be shorter or longer than 4 days is not certain. The lack of responsiveness of GSH could also be connected with responses at other levels of biological organisation such as lysosomal alterations, since it is known that lysosomes in the digestive gland of mussels play a key role in metal sequestration, accumulation, and detoxification (Soto and Marigómez, 1997).

Digestive cell lysosomes are very sensitive to a wide range of contaminants and their responses are widely used as general stress or effect biomarkers of pollution, both in field and laboratory studies (Moore, 1988; Regoli, 1992; Etxeberria et al., 1994; Marigómez et al., 1996; Marigómez and Baybay-Villacorta, 2003; ICES, 2011; Izagirre et al., 2014a). In the present study after 21 days of exposure at 10 µg Cu/ L, membrane destabilisation and lysosomal structural changes were measured. The lysosomal membrane stability test is recommended by the OSPAR Convention to assess the biological effects of contaminants (UNEP/RAMOGE, 1999). Overall, mussels are considered healthy when they exhibit LP values above 20 min and stressed when LP values are below 10 min (Viarengo et al., 2000; Dagnino et al., 2007; Izagirre and Marigómez, 2009). In the present study, low LP values were recorded in controls of the three *Mytilus spp*. Nevertheless, low LP values were also observed in natural conditions at certain moments of the process of intracellular digestion (Tremblay and Pellerin-Massicote, 1997; Izagirre et al., 2009) and in different lysosomal populations of laboratory control *M. edulis* (Moos et al., 2012). In fact, *M. edulis* showed the lowest LP values in the control group. After Cu exposure, only LP was maintained in *M. edulis* and decreased in *M. galloprovincialis* and *M. trossulus*. However, only *M. trossulus* showed a significant decrease in LP value, which was in accordance with the other biomarkers, indicating that *M. trossulus* was the most sensitive species.

The assessment of lysosomal structural changes provides an indication of general environmental stress exhibited by the mussel (Cajaraville et al., 2000; Marigómez and Baybay-Villacorta, 2003). Overall, exposure of mussels to pollutants induces lysosomal enlargement (Moore, 1988; Etxeberria et al.

1994; Marigómez & Baybay-Villacorta, 2003). However, a decrease in lysosomal size was shown after 21 days exposure in *M. edulis* and *M. trossulus*. These results are in accordance with Etxeberria et al., (1994), who observed a significant reduction in Nv of *M. galloprovincialis* following 20 days exposure to 8 µg Cu/L. This response has also been observed in mussels exposed to organic pollutants (Moore, 1998; Cajaraville et al., 1995; Marigómez & Baybay-Villacorta, 2003). In fact, lysosomal size reduction itself appears to be transient since longer exposure times have been found to provoke lysosomal enlargement (Cajaraville et al., 1995).

In addition to chemical stress, lysosomal biomarkers change depending on natural environmental factors, such as temperature (Etxeberria et al., 1995; Tremblay et al., 1998; Izagirre et al., 2014b; Mugica et al., submitted). For instance, lysosomal size and membrane stability show marked seasonal variability, with lysosomes more conspicuous (high Vv, Sv and low S/V values) in the summer than in winter and their membranes are more destabilized (low LP values). Furthermore, low lysosomal responsiveness in winter has been previously reported (Garmendia et al., 2010; Lekube et al., 2014; Mugica et al., submitted). Since the study was performed at 15°C, which was more representative of winter for *M. galloprovincialis* and spring for *M. edulis* and *M. trossulus*, due to their different geographical origins. The mussels were therefore more likely adapted to different seasonal patterns in water temperature and food availability. This could be a reason for the lack of lysosomal responsiveness in *M. galloprovincialis* to Cu exposure and the significant differences with *M. edulis* and *M. trossulus*.

Intracellular neutral lipid accumulation in the lysosomes and cytosol of the digestive cells of mussels has been mainly linked to pollution. However, intracellular neutral lipid accumulation differs between geographical locations and can vary throughout the year, mainly due to changes in the reproductive cycle and food availability (Cancio et al., 1999; Garmendia et al., 2010). The observed differences in Vv_{NL} between *Mytilus spp.* could therefore be due to natural factors such as temperature and reproductive cycle, rather than any physiological and/or cellular differences caused by metal exposure.

Lipofuscins are pigments regarded as the end products of lipid peroxidation (Moore, 1990; Yin, 1996; Terman et al., 1999; Brunk and Terman, 2002). Their accumulation in digestive cells is one of the best documented changes in lysosomal content in response to pollutant exposure, and therefore, lipofuscin

accumulation is considered a general response (Viarengo et al., 1990; Regoli, 1992). In the present study, lipofuscin accumulation followed the same pattern of Vv_{Lys}, with a higher amount of lipofuscins in controls than in exposed groups. This decrease is in accordance with lysosomal enlargement indicating a close relation between these two biomarkers. The reduction of lysosomes and lipofuscins could be explained by cell type replacement and tissue renewal processes, which are typical responses in long term pollutant exposures (Cajaraville et al, 1995; Zaldibar et al., 2007).

It has been shown that the responsiveness of *M. trossulus* was higher when exposed to Cu stress than the other studied species. However, differences between the initial conditions of mussels from the different geographical locations cannot be ignored and such differences were thought to influence, to a certain extent, some of their biological responses. However, in the case of *M. edulis* and *M. trossulus* similar temperatures and seasonal patterns were experienced prior to laboratory acclimation. During the experiment, water temperatures were maintained at 15^{o}C, which was considered to be a compromise between the temperate ranges experienced by the Norwegian mussels and those from the Basque coast. However, the seawater temperature was typical of the summer months in Norway and the winter months on the Basque coast (www.seatemperature.org). Although the long acclimation period of 4-6 weeks was thought to be sufficient time for the normalisation of the physiological parameters (Widdows and Bayne, 1971; Altieri, 2006), there is still a lack of knowledge of the biological responses of mussels with changes in seasonal/thermal patterns. For instance, the use of energy reserves, gonad development and food availability could be important factors, which are known to change with the seasons (Garmendia et al., 2010). Another important factor that could modulate the biomarkers is the age of the mussels. In the present work, mussels of similar size have been used in order to normalise these differences but recent work has confirmed that mussels from different geographical areas could have different growth rates and different biomarker responses (Izagirre et al. 2014b).

In conclusion, it is clear from this study that differences in metal bioaccumulation and biomarker responses occur between the three *Mytilus* species, which may influence the assessment of the environmental health status in marine pollution programmes; particularly knowing that a large extent of geographical areas around the European coast have two or three of the species studied and their

hybrids (Kijewski et al., 2011; Brooks and Farmen, 2013). However, the potential physiological differences between species could be buffered by the adaptation to particular environmental conditions and make similar the responses to pollutants and other environmental stressors. Thus, future research is needed to determine the degree of influence of the species in biological responses to pollutants especially in different mussel species from the same population/sampling point.

5. Acknowledgements

The authors would like to thank the technical staff at the NIVA research station at Solbergstrand, Norway for the set-up and maintenance of the exposure system, as well as the institutional grants to NIVA for funding.

5. References

Ackerman JD, Nishizaki MT. 2004. The effect of velocity on the suspension feeding and growth of the marine mussels *Mytilus trossulus* and *M. californianus*: implications for niche separation. J Marine Syst 49: 195-207.

Akester RJ, Martel AL. 2000. Shell shape, dysodont tooth morphology, and hinge-ligament thickness in the bay mussel *Mytilus trossulus* correlate with wave exposure. Can J Zool,, 78:240-253.

Altieri AH. 2006. Inducible variation in hypoxia tolerance across the intertidal–subtidal distribution of the blue mussel *Mytilus edulis*. Mar Ecol- Prog Ser, 325: 295-300.

Baršienė J, Andreikėnaitė L, Bjornstad A. 2010. Induction of micronuclei and other nuclear abnormalities in blue mussels Mytilus edulis after 1-, 2-, 4- and 8-day treatment with crude oil from the North Sea. Ekologija 56: 124-131.

Bignell, J.P., M. J. Dodge, S. W. Feist, B. Lyons, P. D. Martin, N. G. H. Taylor, D. Stone, L. Travalent & G. D. Stentiford. 2008. Mussel histopathology: effects of season, disease and species. Aquat. Biol. 2:1-15.

Bolognesi C, Fenech M. 2012. Mussel micronucleus cytome assay. Nature America. 7: 1125-1137.

Brooks SJ, Farmen E. 2013. The distribution of the mussel *Mytilus* species along the Norwegian coast. Journal of Shellfish Research, 32:1-6.

Brooks, S. J., C. Harman, M. Grung, E. Farmen, A. Ruus, S. Vingen, B. F. Godal, J. Baršienė, L. Andreikėnaitė, H. Skarphéðinsdóttir, B. Liewenborg &, R. C. Sundt. 2011. Water column monitoring of the biological effects of produced water from the Ekofisk offshore oil installation from 2006 to 2009. J. Toxicol. Environ. Health. 74:582-604.

Brunk UT, Terman A. 2002. Lipofuscin: mechanisms of age-related accumulation and influence on cell function. Free Radical Biol Med, 33: 611-619.

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci Total Environ, 247: 295-311.

Cajaraville MP, Marigómez JA, Angulo E. 1989. A stereological survey of lysosomal structure alterations in *Littorina littorea* exposed to 1-naphtol. Comp Biochem Physiol: 93C: 231–7.

Cajaraville MP, Robledo Y, Etxeberria M & Marigómez I. 1995. Cellular biomarkers as useful tools in the biological monitoring of environmental pollution: molluscan digestive lysosomes. In: Cajaraville MP (Eds.) Cell biology in environmental toxicology. Bilbo, University of the Basque Country, Press Service, 29-55 pp.

Cancio I, Ibabe A & Cajaraville MP. 1999. Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels *Mytilus galloprovincialis* and its relationship with the lipid content. Comp Biochem Physiol C: Comp Pharmacol Toxicol, 123, 135-144.

Canesi L, Viarengo A, Leonzio C, Filippelli M, Gallo G. 1999. Heavy metals and glutathione metabolism in mussel tissues. Aquatic Toxicology, 46:(1)67–76

Christensen JH, Glasius M, Pécseli M, Platz J, Pritzl G. 2002. Polybrominated diphenyl ethers (PBDEs) in marine fish and blue mussels from southern Greenland. Chemosphere, 47: 631-638.

Coustau, C., F. Renaud, C. Maillard, N. Pasteur & B. Delay. 1991. Differential susceptibility to a trematode parasite among genotypes of *Mytilus edulis/galloprovincialis* complex. Genet. Res. 57:207-212.

Culling CFA. 1974. Handbook of histopathological and histochemical techniques, 3rd edition. Buttherworths, Guildford, 712 pp.

Dagnino A, Allen JI, Moore MN, Broeg K, Canesi L, Viarengo A. 2007. Development of an expert system for the integration of biomarker responses in mussels into an animal health index. Biomarkers, 12: 155-172.

Damiens G, Gnassia-Barelli M, Loquès F, Roméo M, Salbert V. 2007. Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere: 66:574-583.

Davies IM, Vethaak AD. 2012. Integrated marine environmental monitoring of chemicals and their effects. ICES cooperative research report. No. 315, 277 pp.

Dietz R, Riget F, Cleemann M, Aarkrog A, Johansen P, Hansen JC. 2000. Comparison of contaminants from different trophic levels and ecosystems. Sci Total Environ, 245: 221-231.

Etxeberria M, Cajaraville MP & Marigómez I. 1995. Changes in digestive cell lysosomal structure in mussels as biomarkers of environmental stress in the Urdaibai estuary (Biscay Coast, Iberian Peninsula). Marin Poll Bull, 30: 599-603.

Etxeberria M, Sastre I, Cajaraville MP, Marigómez I. 1994. Digestive lysosome enlargement induced by experimental exposure to metals (Cu, Cd and Zn) in mussels collected from a zinc-polluted site. Arch Environ Contam Toxicol,27: 338-345.

Garmendia L, Soto M, Cajaraville MP, Marigómez I. 2010. Seasonality in cell and tissue-level biomarkers in *Mytilus galloprovincialis*: relevance for long-term pollution monitoring. Aquatic Biol 9, 203–219.

Goldberg ED, Bowen VT, Farrington JW, Harvey G, Martin JH, Parker PL, Risebrough RW, Robertson W, Schneider E, Gamble E. 1978. The Mussel Watch. Environmental Conservation, 5, pp 101-125.

Hilbish, T. J., E. W. Carson, J. R. Plante, L. A. Weaver & M. R. Gilg. 2002. Distribution of *Mytilus edulis, M. galloprovincialis,* and their hybrids in open-coast populations of mussels in south western England. Mar. Biol. 140:137-142.

Hylland K, Tollefsen KE, Ruus A, Jonsson G, Sundt RC, Sanni S, Roe Utvik TI, Johnsen S, Nilssen I, Pinturier L, Balk L, Barsiene J, Marigomez I, Feist SW, Borseth JF. 2008. Water column monitoring near oil installations in the North Sea 2001-2004. Mar. Pollut. Bull. 56: 414-429.

ICES/OSPAR SGIMC, 2011. Report of the study group on integrated monitoring of contaminants and biological effects (SGIMC). 14-18 March 2011. Copenhagen, Denmark ICES CM 2011/ACOM:30.

Inoue, K., H. Waite, M. Matsuoka, S. Odo & S. Harayama. 1995. Interspecific variations in adhesive protein sequences of *Mytilus edulis*, *M. galloprovincialis*, and *M. trossulus*. Biol. Bull. 189:370-375.

Izagirre U, Errasti A, Bilbao E,Múgica M, Marigómez I, 2014a. Combined effects of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70in mussels, *Mytilus galloprovincialis*. Aquat Toxicol 149, 145-156.

Izagirre U, Garmendia L, Soto M, Etxebarria N, Marigómez I. 2014b. Health status assessment through an integrative biomarker approach in mussels of different ages with a different history of exposure to the Prestige oil spill.

Izagirre U, Marigómez I. 2009. Lysosomal enlargement and lysosomal membrane destabilisation in mussel digestive cells measured by an integrative index. Environ Pollut 157, 1544-1553.

Izagirre U, Ruiz P Marigómez I. 2009. Time-course study of the early lysosomal responses to pollutants in mussel digestive cells using acid phosphatase as lysosomal marker enzyme. Comp Biochem Physiol C: Comp Pharmacol Toxicol 149, 587-597.

Kamenic H, Lyon A, Paterson PG, Juurlink BHJ, 2000. Monochlorobimane fluorometric method to measure tissue glutathione. *Analytical Biochemistry*, 286: 35-37.

Kijewski, T., B. Śmietanka, M. Zbawicka, E. Gosling, H. Hummel & R. Wenne. 2011. Distribution of *Mytilus* taxa in European coastal areas as inferred from molecular markers. J. Sea Res. 65:224–234.

Lam PKS & Gray JS. 2003. The use of biomarkers in environmental monitoring programmes. Marine Pollution Buletin:46(2):182-186.

Lehtonen KK & Schiedek D. 2006. Monitoring biological effects of pollution in the Baltic Sea: Neglected—but still wanted? Marine Poll. Bull. 53: 377-386.

Lekube X., Izagirre U., Soto M., Marigómez I. 2014. Lysosomal and tissue-level biomarkers in mussels cross-transplanted among four estuaries with different pollution levels. Sci Total Environ, 472: 36–48.

Lobel, P. B., S. P. Belkhode, S. E. Jackson & H. P. Longerich. 1990. Recent taxonomic discoveries concerning the mussel *Mytilus*: Implications for biomonitoring. Arch. Environ. Contam. Toxicol. 19:508-512.

Lowe DM, Moore MN, Clarke RK. 1981. Effects of oil on digestive cells in mussels: Quantitative alterations in cellular and lysosomal structure. Aquatic Toxicology, 1: 213–226.

Marigómez I, Baybay-Villacorta L. 2003. Pollutant specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. Aquat Toxicol, 64: 235-257.

Marigómez I, Garmendia L, Soto M, Orbea A, Izagirre U & Cajaraville MP. 2013. Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige oil spill ‘‘Mussel Watch’’. Ecotoxicology 22: 486-505.

Marigómez I, Orbea A, Olabarrieta I, Etxeberria M, Cajaraville MP. 1996. Structural changes in the digestive lysosomal system of sentinel mussels as biomarkers of environmental stress in musselwatch programmes. Comp Biochem Physiol, 113C: 291-297.

Moore MN. 1988. Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscan digestive cells to environmental and experimental exposure to xenobiotics. Mar Ecol-Prog Ser, 46: 81-89.

Moore MN. 1990. Lysosomal cytochemistry in marine environmental monitoring. Histochem. J.22: 187-191.

Moore MN. 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel Mytilus edulis, and changes induced by thermal stress. Cell Tissue Res, 175:279–87.

Moos N, Burkhardt-Holm, Köhler A. 2012. Uptake and Effects of Microplastics on Cells and Tissue of the Blue Mussel *Mytilus edulis* L. after an Experimental Exposure. Environ. Sci. Technol., 46, 11327−11335

Mubiana VK, Vercauteren K, Blust R. 2006. The influence of body size, condition index and tidal exposure on the variability in metal bioaccumulation in *Mytilus edulis*. Environmental Pollution, 144(1)272-279.

Mugica M, Sokolova IM, Izagirre U, Marigómez I. Season-dependent effects of elevated temperature on stress biomarkers, energy metabolism and gamete development in mussels. Submitted in Mar Environ Res.

Pearse AGE. 1972. Appendix 26. In Pearse AGE. Histochemistry, Theoretical and Applied. Edinburgh, New York, Churchill Livingstone, 1383-1385.

Rainbow PS, 2007. Trace metal bioaccumulation: Models, metabolic availability and toxicity. Environment International. 33(4):576-582.

Regoli F, & Principato G. 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, Mytilus galloprovincialis, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquatic Toxicology. 31(2):143-164.

Regoli F. 1992. Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution. Mar Ecol Prog Ser, 84: 63-69.

Seed R. 1968. Factors Influencing Shell Shape in the Mussel *Mytilus edulis*. J. Mar. Biol. Assoc. U. K., 48: 561-584.

Sokal FJ, Rohlf RR. 1995. Biometry. The principles and practice of statistics in biological research. 3rd edition. Nueva York: Freeman and Company WH, p. 887.

Soto M, Marigómez I. 1997. BSD Extent, an Index for Metal Pollution Screening Based on the Metal Content within Digestive Cell Lysosomes of Mussels as Determined by Autometallography. Ecotox Environ Safe, 37: 141-151.

Terman A, Abrahamsson N, Brunk UT. 1999. Ceroid/lipofuscin-loaded human fibroblasts show increased susceptibility to oxidative stress. Exp Gerontol, 34: 755-770.

Tremblay R, Pellerin Massicotte J. 1997. Effect of the tidal cycle on lysosomal menbrane stability in the digestive gland of *Mya arenaria* and *Mytilus* (L.). Comp Biochem Physiol 117A: 99-104.

Tremblay, R., Myrand, B., Guderley, H. 1998. Temporal variation of lysosomal capacities in relation to susceptibility of mussels, *Mytilus edulis*, to summer mortality. Mar Biol, 132, 641–649.

UNEP/RAMOGE. 1999. Manual on the biomarkers recommended for the MED POL biomonitoring programme. UNEP, Athens, UNEP. Athens.

Väinölä, R. & P. Strelkov. 2011. *Mytilus trossulus* in Northern Europe. Mar. Biol. 158: 817-833.

Viarengo A, Canesi L, Pertica M, Poli G, Moore MN, Orunesu M.1990. Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. Comp Biochem Physiol C: Comp Pharmacol Toxicol,, 97: 37-42.

Viarengo A, Lafaurie M, Gabrielides GP, Fabbri R, Marro A, Roméo M. 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. Mar Environ Res, 49: 1-18.

Widdows J, Bayne BL. 1971. Temperature Acclimation of *Mytilus edulis* With Reference to its Energy Budget. J. Mar. Biol. Assoc. U. K., 51: 827-843.

Yin D. 1996. Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. Free Radic Biol Med, 21: 871-888.

Zaldibar B, Cancio I, Soto M, Marigómez I. 2007. Reversible alterations in epithelial cell turnover in digestive gland of winkles (*Littorina littorea*) exposed to cadmium and their implications for biomarker measurements. Aquat Toxicol 81: 183-196.

Žegura B, Lah TT, Filipič M. 2006. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 611: 25-33.

Table 1. The numbers of the various *Mytilus* complex species within the collected populations.

Mytilus complex	Bilbao (n=85)	Outer Oslo fjord (n=78)	Tingvoll fjord nr Molde, Norway (n= 73)
M. galloprovincialis	79 (93%)		
M. edulis		74 (95%)	1 (1%)
M. trossulus	1 (1%)		56 (77%)
M. galloprovincialis/ M. edulis hybrid	5 (6%)	1 (1%)	
M. galloprovincialis/ M. trossulus hybrid			3 (4%)
M. edulis/ M. trossulus hybrid		3 (4%)	13 (18%)

NB: only the pure *Mytilus* were selected for bioaccumulation and biomarker measurements.

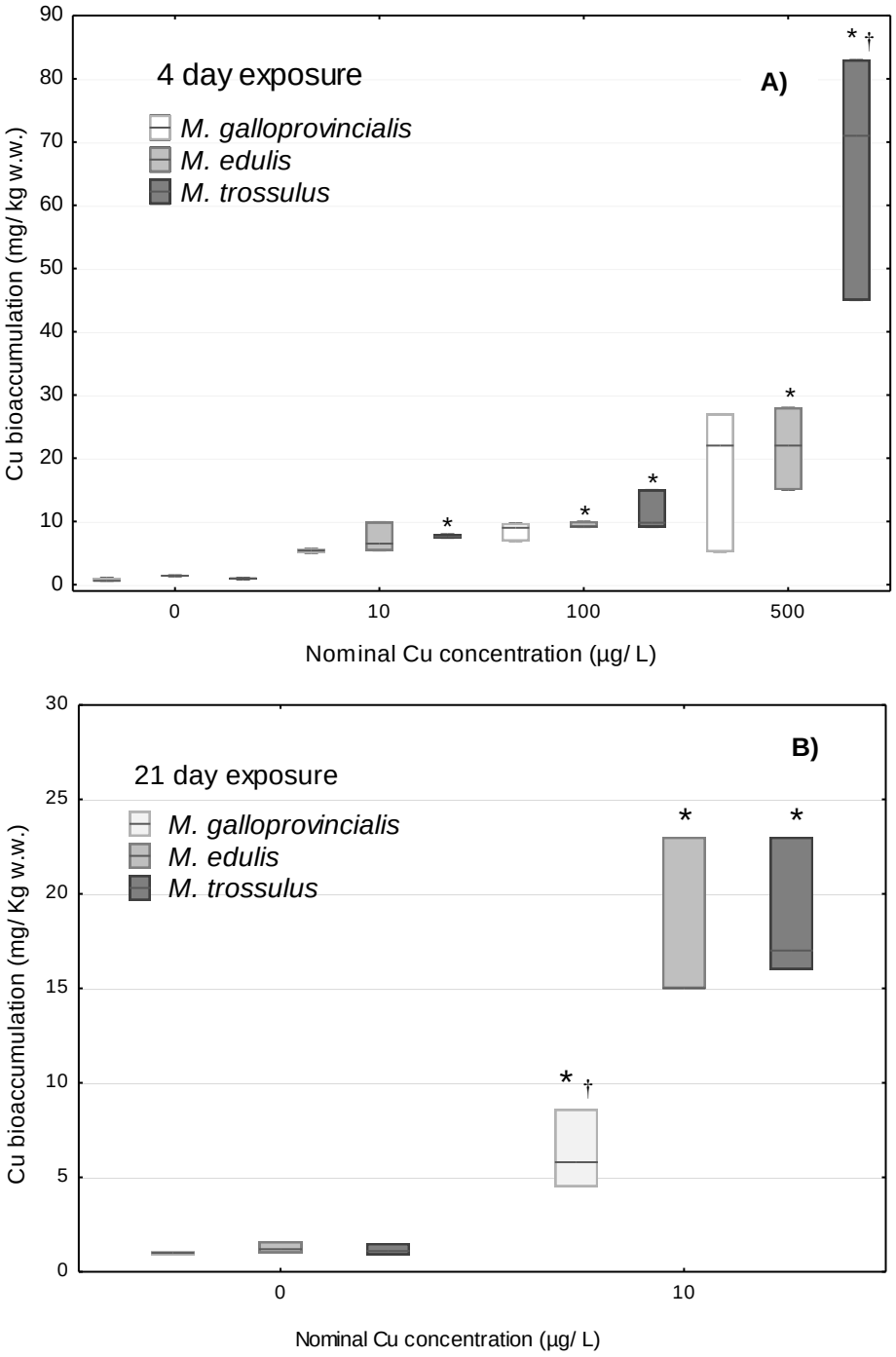

Figure 1. The bioaccumulation of copper measured in individual whole mussel homogenates of the different *Mytilus spp*. following an A) 4 day and a B) 21 day exposure to nominal copper concentrations (median ± quartiles, n= 3). * Significantly different from control group (0 Cu); † significant difference from other *Mytilus* within treatment group.

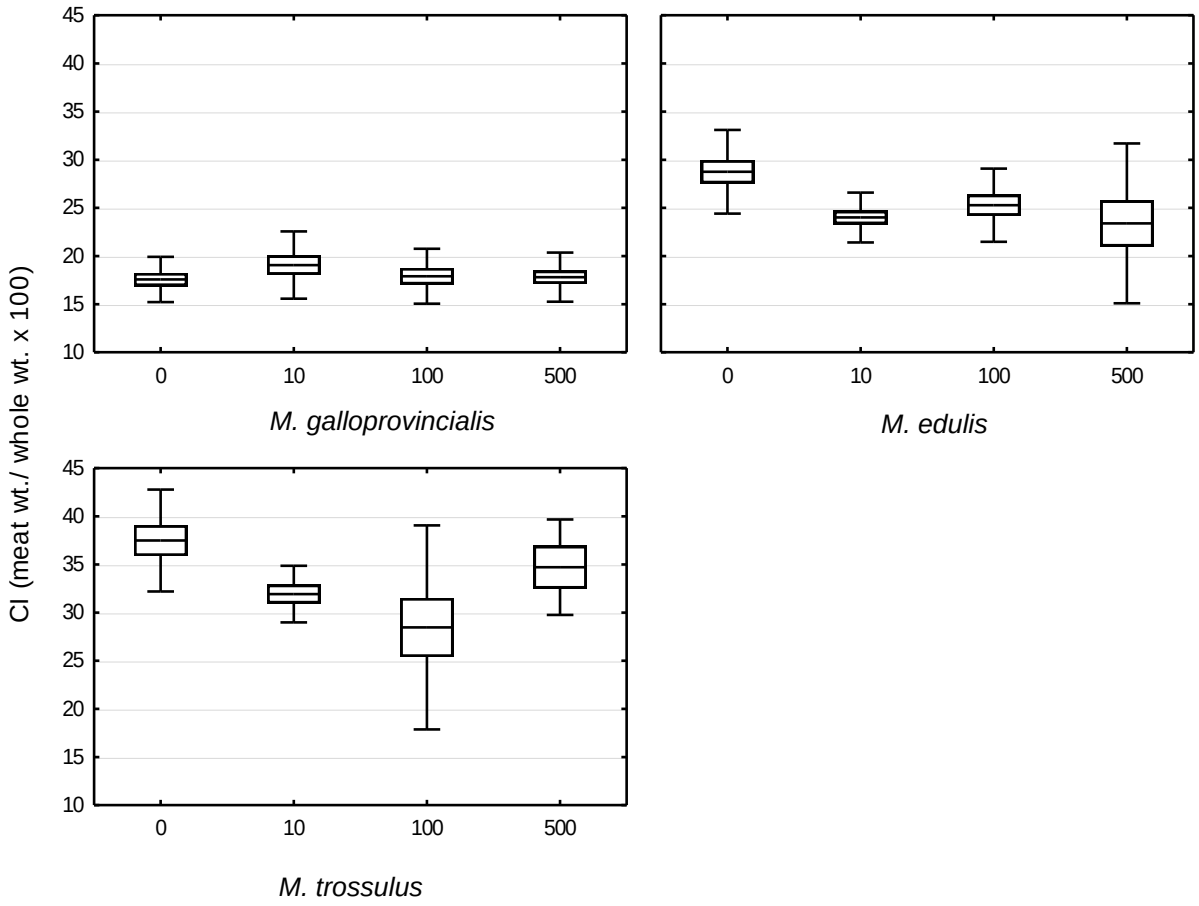

Figure 2. Condition index of the three *Mytilus* spp. following 4 day exposure to nominal copper concentrations (mean, SD (box) ± SE (outer line), n=10).

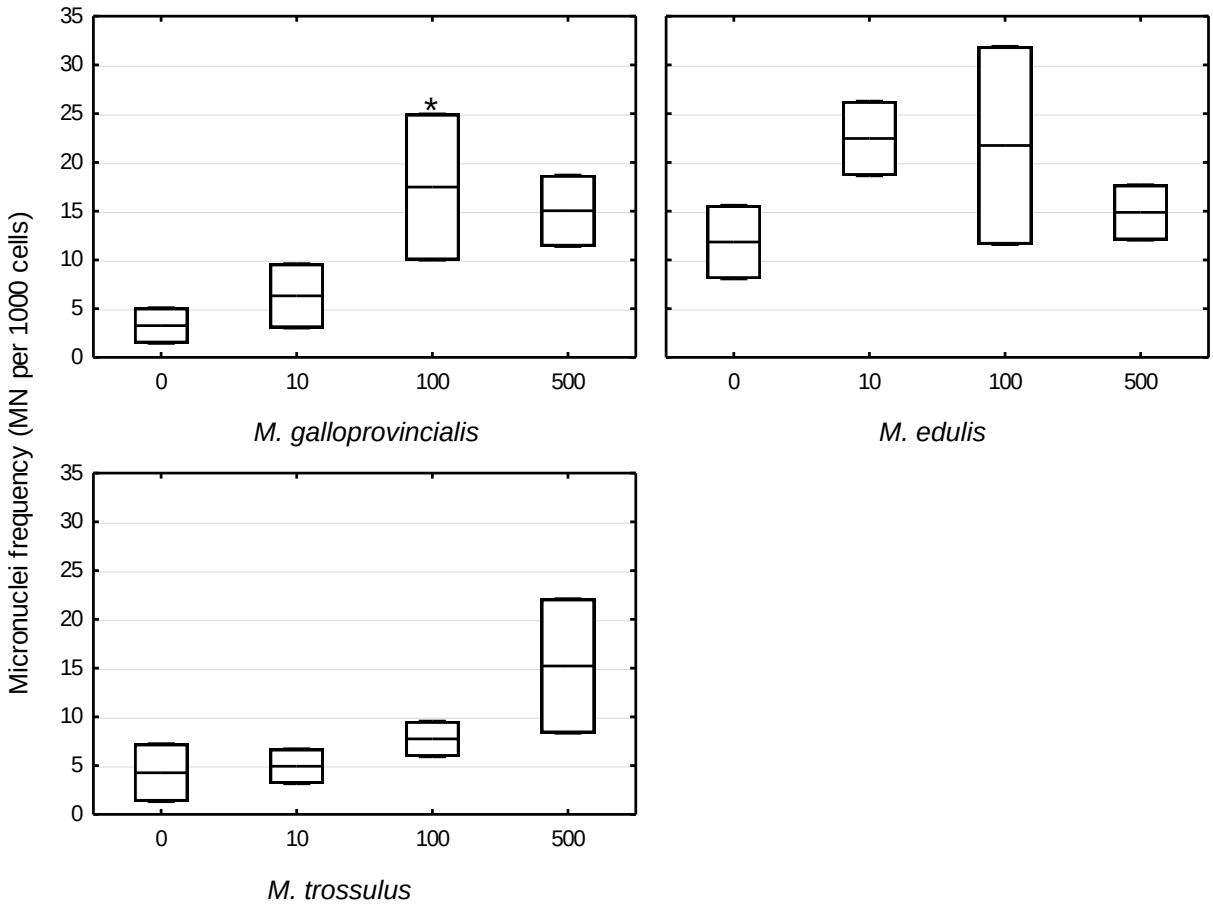

Figure 3. The frequency of micronuclei in haemocytes of the three *Mytilus* species following 4 day exposure to nominal copper concentrations (µg/ L, mean ± SE, n=10). * significantly different from control group.

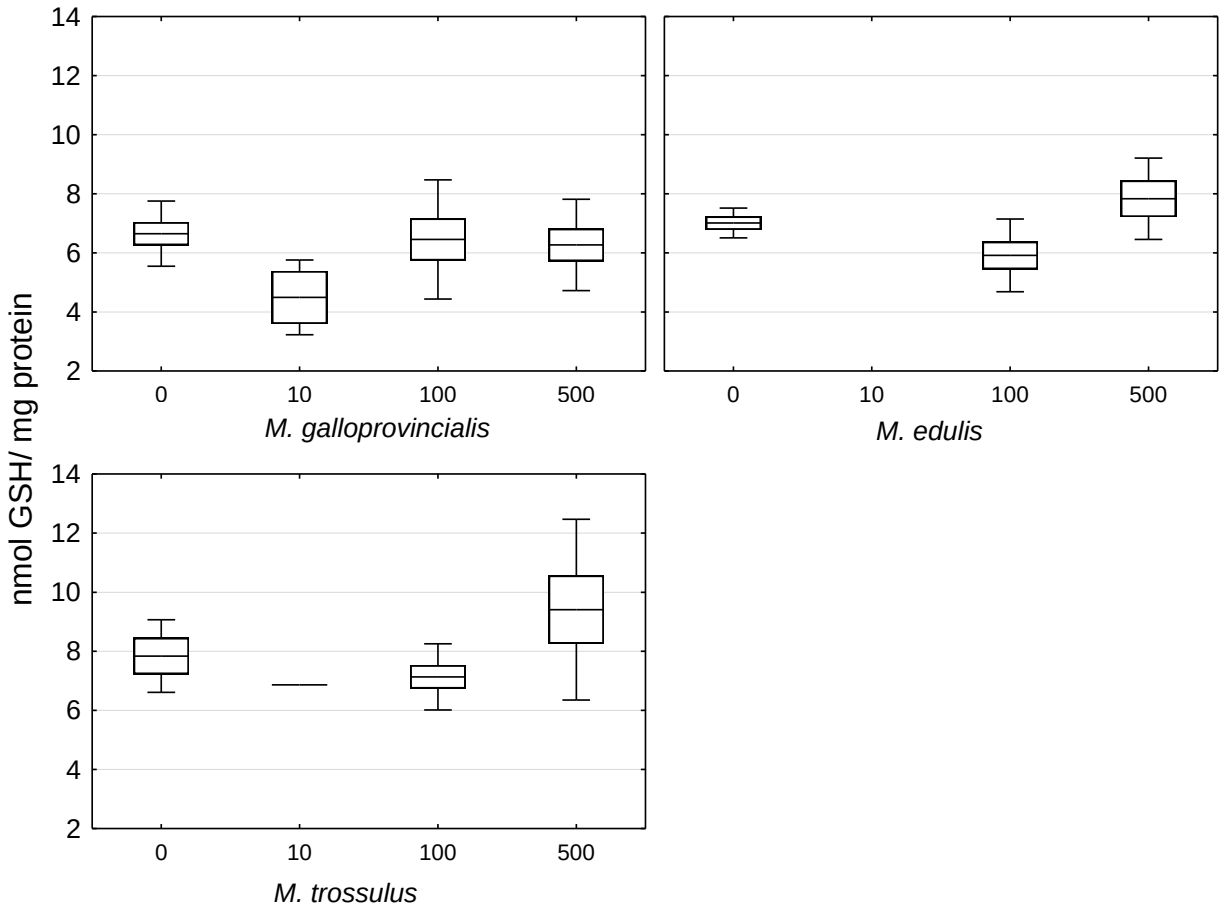

Figure 4. Glutathione in digestive gland samples of the three *Mytilus* species following a 4 day exposure to nominal copper concentrations (µg/ L, mean, SD (box) ± SE (outer line), n=10). Note GSH was note measured in *M. edulis* at 10µg /L.

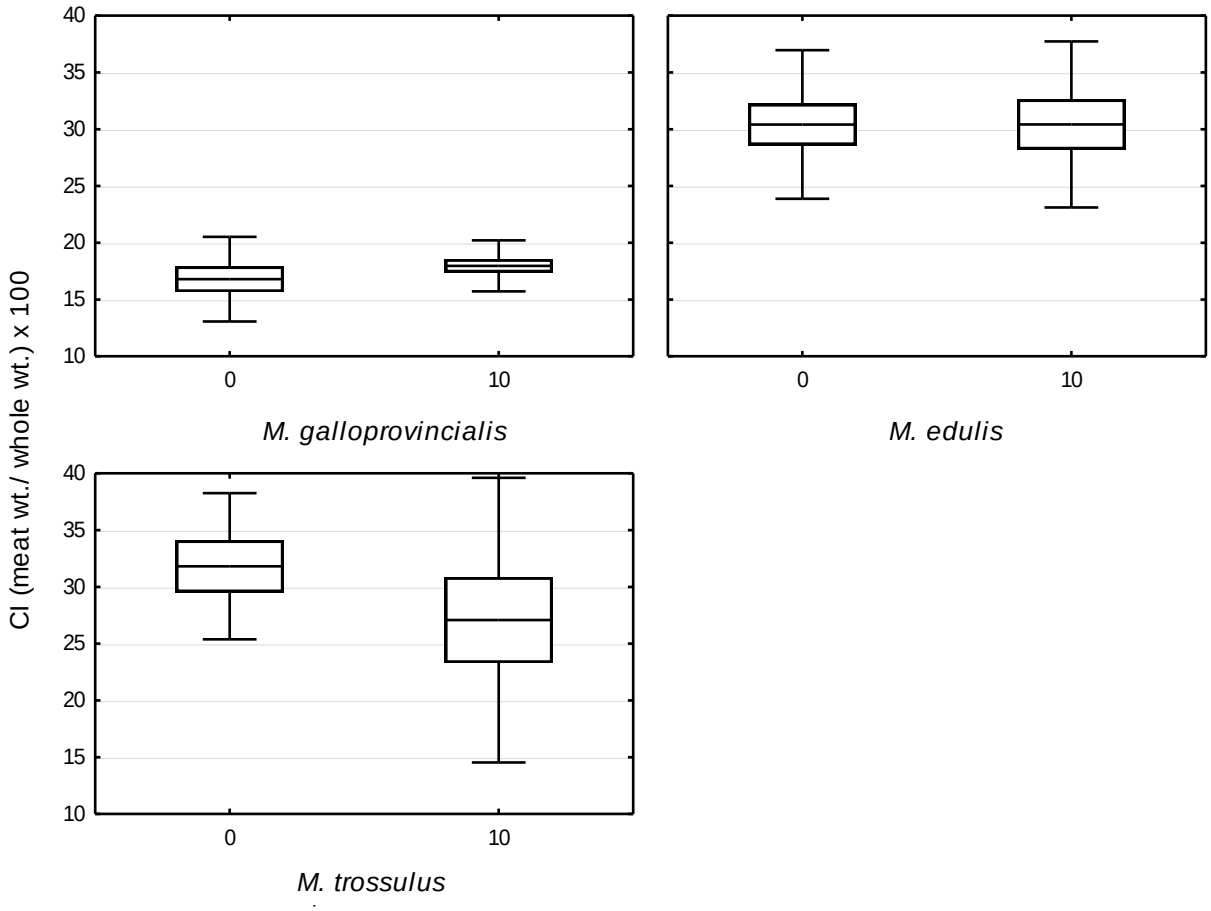

Figure 5. Condition index of the three *Mytilus* spp. following 21 day exposure to nominal copper concentrations (µg/ L, mean, SD (box) ± SE (outer line), n=10).

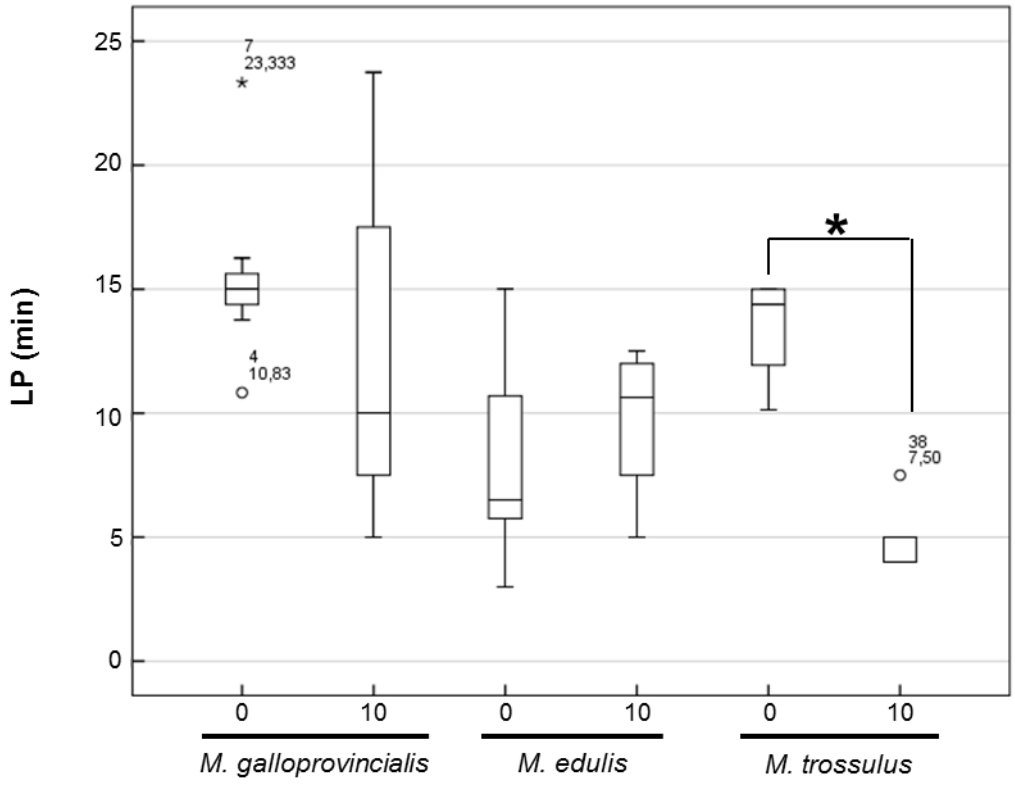

Figure 6. Lysosomal membrane stability (LMS) measured as labilisation period (LP) in the digestive gland cells of the three *Mytilus* spp. following 3 week nominal copper exposure (µg/ L Cu). Intervals indicate standard deviation, asterisks indicate significant differences among control and exposed mussel of the same species according to the Man-Whitney U-test ($p<0.05$).

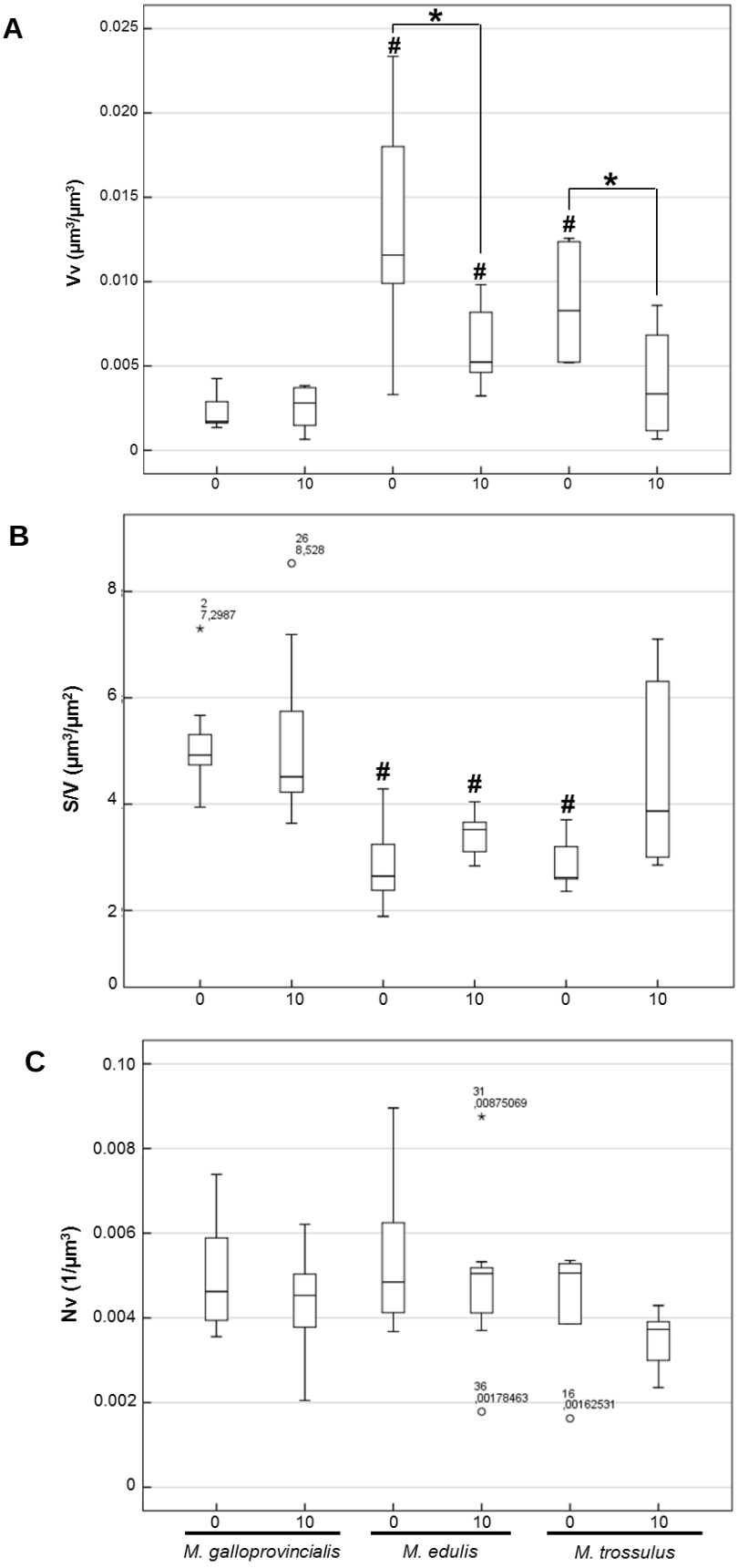
A
B
C
Vv (µm³/µm³)
S/V (µm³/µm²)
Nv (1/µm³)
0.025
0.020
0.015
0.010
0.005
0
8
6
4
2
0
0.10
0.008
0.006
0.004
0.002
0
10
*
#
26
8,528
2
7,2987
31
,00875069
36
,00178463
16
,00162531
M. galloprovincialis
M. edulis
M. trossulus

Figure 7. Lysosomal structural changes in digestive gland of the three *Mytilus spp*. following 21 day nominal copper exposure (µg/ L Cu). (A) Lysosomal volume density (VvL); (B) lysosomal numerical density (Nv); and (C) lysosomal surface to volume ratio (S/VL). Intervals indicate standard deviation, asterisks indicate significant differences among control and exposed mussel of the same species according to the Duncan's test performed after one-way ANOVAs ($p<0.05$) and hashes indicate significant differences of *M. edulis* and *M. trossulus* respecting *M. galloprovincialis* in each experimental condition according to the Student t-test ($p<0.05$).

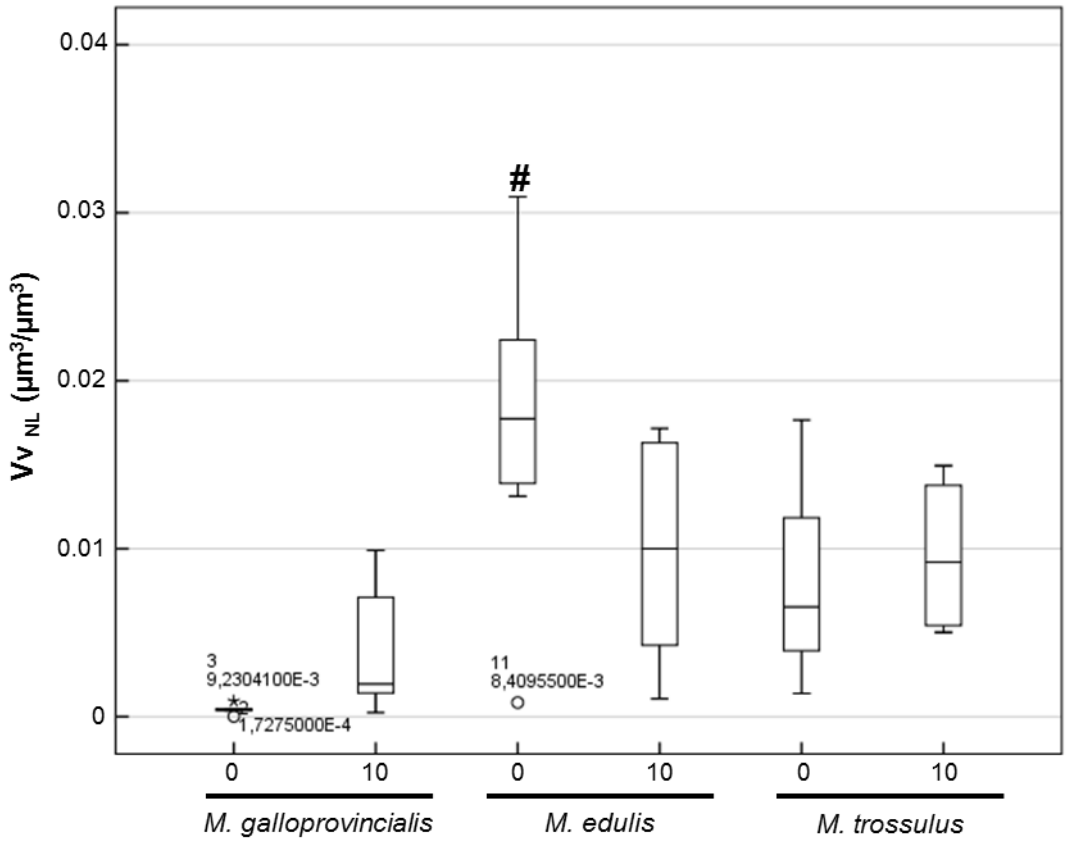

Figure 8. Intracellular neutral lipid volume density (VvNL) in digestive gland of the three *Mytilus spp*. following 21 day copper exposure (µg/ L Cu). Intervals indicate standard deviation and hashes indicate significant differences of *M. edulis* and *M. trossulus* respecting *M. galloprovincialis* in each experimental condition according to the Student t-test ($p<0.05$).

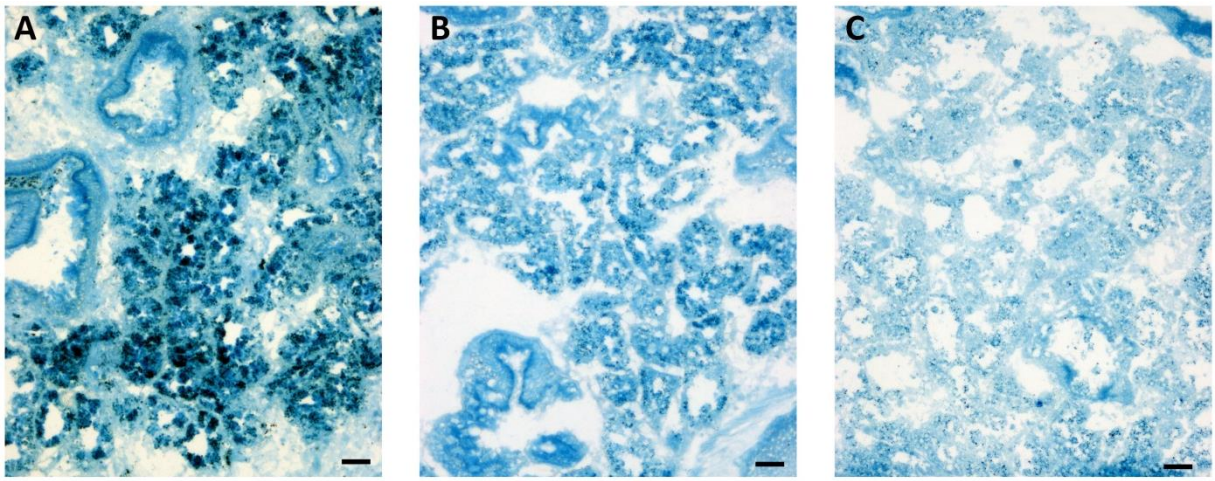

Figure 9. Histochemistry of lipofuscins in digestive gland of mussels. (A) Control *M. galloprovincialis*; (B) Control *M. edulis*; (C) 10 µg Cu/ L exposed *M. edulis* for 21 days. Scale bar = 50 µm.