EFFECT OF COPPER MINE TAILINGS ON LIPID PEROXIDATION, DNA DAMAGE AND HISTOPATHOLOGY IN SOFT-SHELL CLAMS (*MYA ARENARIA*) AT LITTLE BAY, NEWFOUNDLAND

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EFFECT OF COPPER MINE TAILINGS ON LIPID PEROXIDATION, DNA DAMAGE AND HISTOPATHOLOGY IN SOFT-SHELL CLAMS (*MYA ARENARIA)* AT LITTLE BAY, NEWFOUNDLAND

by

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ABSTRACT

Hamoutene, D., Payne, J.F., French, B., Veinott, G. and Fancey, L. 2002. Effect of copper mine tailings on lipid peroxidation, DNA damage and histopathology in soft-shell clams (*Mya arenaria)* at Little Bay, Newfoundland. Can. Tech. Rep. Fish. Aquat. Sci. 2436: iv + 12 p.

In 1989 the tailings pond dam at the site of a former copper mine near Little Bay, Newfoundland ruptured and tailings spilled into Little Bay arm. As a result, the Little Bay marine area has become contaminated with heavy metals (Cu, Ni, Zn, Fe, Mn...). Some metals can generate free radicals and affect macro-molecules including lipids, proteins and DNA. We assessed the extent of lipid peroxidation (TBARS), DNA damage and catalase activity (an antioxidant enzyme) in clams collected for two consecutive years from the Little Bay site and two reference sites. Detailed histopathological studies were also carried out. The Comet assay was used to assess DNA breakage and showed no differences between clams from reference and mining sites. Histopathological studies of hepatopancreatic, gonadal and gill tissues also revealed no differences between sites. TBARS levels measured in clams from the mining site were higher the first sampling year but not the second one. Catalase activity was higher the second year suggesting that it may have influenced TBARS levels. These results indicate that mine tailings have potential to trigger an increase in lipid peroxidation in animals living in these tailings but that this increase can be reduced therefore suggesting no major toxicity. This is also supported by the histopathology studies. The results overall are of interest with respect to options for environmental disposal of tailings enriched in copper. Our study also demonstrates the successful application of a biochemical sexing method used for mussels to clam specimens.

RÉSUMÉ

Hamoutene, D., Payne, J.F., French, B., Veinott, G. and Fancey, L. 2002. Effect of copper mine tailings on lipid peroxidation, DNA damage and histopathology in soft-shell clams (*Mya arenaria)* at Little Bay, Newfoundland. Can. Tech. Rep. Fish. Aquat. Sci. 2436: iv + 12 p.

En 1989, la rupture d'un barrage isolant une mine de cuivre désaffectée à Little Bay, Terre Neuve, provoque le déversement des rejets dans l'eau de mer. Par conséquent, le bras de mer mitoyen est contaminé par des métaux lourds (Cu, Ni, Zn, Fe, Mn...) dont certains peuvent produire des radicaux libres pouvant affecter les lipides, les protéines et l'ADN. Nous avons donc évalué la peroxydation lipidique, les dommages causés à l'ADN et le niveau d'activité d'une enzyme antioxydante: la catalase chez des palourdes *Mya arenaria* prélevées pendant 2 années consécutives au site contaminé ainsi qu'au niveau de 2 sites de référence. De même, une étude histopathologique de l'hepatopancréas, des gonades et des branchies a été réalisée.

Le test "Comète" utilisé pour évaluer les dommages de l'ADN n'a montré aucune différence entre les échantillons des sites contaminé et de référence, résultat confirmé par l'absence de dommages au niveau des tissus observés. La peroxydation lipidique des échantillons du site contaminé est supérieure à celle du site de référence uniquement la première année de prélèvement, alors que l'activité catalase présente des valeurs plus élevées au site minier que la seconde année. Ceci pourrait suggérer un effet de l'enzyme antioxydante sur les niveaux de peroxydation lipidique. Les résultats de cette étude montrent que la présence des rejets miniers peut déclencher un stress oxydatif chez les palourdes mais que cette réponse est réversible suggérant ainsi aucune toxicité majeure. Les résultats de cette étude ouvre des perspectives quant aux options possibles en matière d'utilisation du milieu marin pour le dépôt de déchets miniers. Notre étude montre également la possible application d'une méthode biochimique de determination du sexe utilisée traditionnellement pour les moules à la reconnaissance des sexes chez la mye.

Introduction

The processing of ore at mining sites generates large amounts of solid wastes (tailings) which contain residual concentrations of all metals originally present in the ore. As part of a study to investigate the impact of mining effluents, clams were collected for two consecutive years from an abandoned copper ore mine in Little Bay, Newfoundland. Little Bay mine, an old mining site in operation in the 1800's, was reactivated from 1961 to 1969 generating a total of 1.8 million tonnes of tailings which were placed in an impoundment area behind a dam in a shallow mixed marine/freshwater bay. The tailings dam was breached and the tailings now form a deltaic beach (Collins and Legrow, 1986) where our sampling took place. The soft-shell clam *Mya arenaria*, a suspension-feeding bivalve found at 15-25 cm depth in the sediment is in direct contact with the tailings and was therefore used as a sentinel organism to assess biological consequences of chronic metal exposure.

Metals can provoke increased production of intermediates of oxygen reduction, such as superoxide radicals and hydrogen peroxide, and generate a prooxidant status in the cell. It has been clearly established that the health of aquatic organisms exposed to different pollutants may be dramatically comproised by disorders associated with oxidative stress (Di Giulio *et al.*, 1989). In this study, the extent of lipid peroxidation as well as DNA and general tissue and organ damage were assessed in *Mya arenaria* specimens. One of the most frequently used method to determine the extent of membrane lipid peroxidation is the Thiobarbituric Acid (TBA) assay (Buege and Aust, 1978) which was chosen for this study. DNA damage was quantified using the Comet assay where electrophoresis under alkaline conditions produces nuclei with increased length or 'tail' proportional to the damage (Singh *et al.*, 1988; Olive *et al.*, 1990). Catalase activity was also measured to determine if this key enzyme involved in antioxidant processes (Livingstone *et al.*, 1992) was affected by mining effluents. Tissue and organ pathology was examined by light microscopy.

Materials and methods

Site locations

Clams (*Mya arenaria*) ranging in size from 50-80 mm were collected from two locations in the western portion of Notre Dame Bay in October 1999 : Little Bay (mining site) and Smith's Harbour (reference site) (Fig.1). In November 2000, clams were collected again from Little Bay but specimens found at Smith's Harbour were small (length <10 mm). Another reference site was therefore selected in Placentia Bay (Swift Current) and clams 50-80 mm long were sampled. Samples from each respective site were held on ice in coolers, transported to the lab and placed in separate aquaria within 72 hours. Tissue samples for biochemical analysis were taken within 48 to 72 hours of arrival at the laboratory.

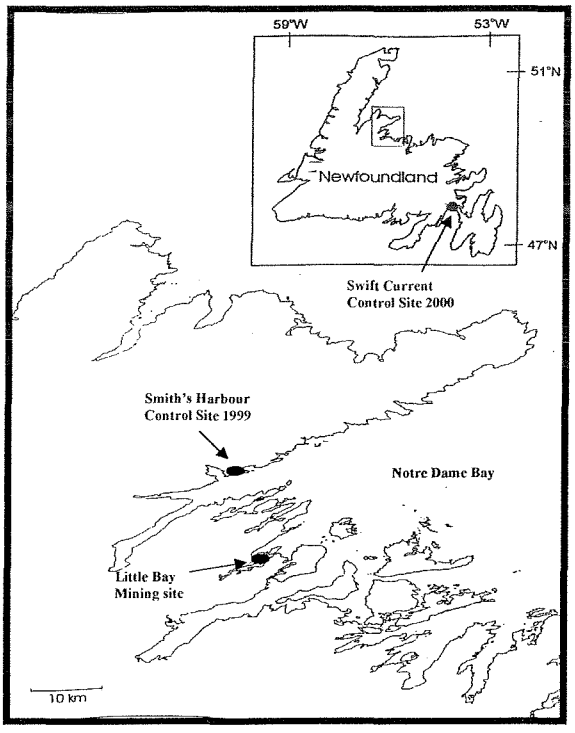

Figure 1- Sampling site locations

Sex determination

Clams between 50-80 mm in length were chosen so all individuals were sexually mature. The method used for sex determination involved heating biopsy samples with a thiobarbituric acid (TBA) reagent (Jabbar and Davies, 1987). Histological sections of gonads were further examined to confirm determination of sex.

Lipid peroxidation and catalase assays

Gills and hepatopancreas of clams were used to prepare S9 for biochemical analysis. A 25% (weight/volume) organ homogenate in 0.1 M phosphate buffer, pH 7.4, was prepared using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10 000 g for 10 min and the supernatant (S9) was stored at -70°C prior to use.

Lipid peroxidation in the gill and hepatopancreas samples was monitored by measuring TBA Reactive Species (TBARS) as described by Uchiyama and Mihara (1978). Tetramethoxypropane was used as an external standard. The level of TBARS is expressed as nmol/mg protein. Catalase activity was measured according to Greenwald (1985) and expressed as μmol H_2O_2 decomposed/min/mg. Protein concentration was determined according to Lowry *et al.* (1951).

The Comet assay

The Comet assay (Steinert *et al.*, 1998) was performed on hemocytes and digestive gland cells of clams collected from Little Bay and Swift Current in November 2000. Hemolymph was withdrawn from adductor muscles and transferred to microfuge tubes and centrifuged for 2 min to pellet the cells (7000g). Digestive glands were excised from each animal and cells were isolated by non-enzymatic tissue dissociation. The use of trypsin for cell disaggregation was omitted due to increase in the % DNA in the tail with this enzyme (Birmelin *et al.*, 1998). The glands were dissociated by stirring pieces of tissue (1g/25 ml) in a buffer containing 20 mM Hepes, 500 mM NaCl, 12.5 mM KCl and 5 mM EDTA (1100 mOsm) for 1hour. The number of cells and their viability was evaluated by Trypan blue exclusion. The Comet assay was conducted as described by Steinert *et al.* (1998) with minor modifications. The DNA unwinding time used was 30 minutes and the samples were subjected to electrophoresis at 25V for 15 minutes. Slides were observed using a fluorescence microscope and ocular micrometer. The total length of the DNA migration was measured for 15-40 cells per slide.

To our knowledge, the Comet assay has never been performed on clam cells. Therefore, we first tested the reproducibility and general conditions of the assay on both hemocytes and hepatopancreas. To obtain cells from digestive glands, organs were incubated for one hour in buffer for tissue dissociation in the absence of trypsin. The dissociation time was based on previous results obtained for mussel digestive gland (unpublished data). The mortality percentage obtained after one hour was 22.7 ± 9.5% (n=4 individuals) which is a high value but acceptable for further testing.

Metal analysis

Samples of the soft-shell clam were collected from Little Bay and Smith's Harbour in Notre Dame Bay in October 1999. Clams were depurated for 24 hours in clean sea water then frozen and transported to the laboratory. At the laboratory, clams were thawed and shucked using plastic implements. Owing to visible tailings contamination, clams had the sheath covering their siphon and their guts removed and discarded. Groups of five individuals were selected at random and placed in trace metal clean containers. The soft tissue was then dried at 100°C to a constant weight. Next, the samples were dry ashed at 500°C and the ash was then digested in concentrated nitric acid. The resulting solution was diluted and analyzed for copper content by ICP-MS. The accuracy of our results was confirmed by comparing the results from the National Institute of Standards and Testing (NIST) standard reference material (SRM) number 2976 to its certified values.

Clam histopathology

Ten clams approximately 5 cm in length were examined from each of the reference and exposed sites. Hepatopancreatic, gill and gonadal tissues were fixed in 10% buffered formalin, and processed in paraffin by conventional histological methods for examination by light microscopy (Lynch *et al.*, 1969). Tissue sections were cut at a thickness of 6 microns and stained in hematoxylin and eosin. Tissues were examined for histological differences at various levels of magnification.

Results

Sex determination

Sex was determined (Table 1) to ensure that it had no effect on TBARS, catalase activity or DNA damage levels measured in clams collected from different sites. The colorimetric biochemical method for sex determination described by Jabbar and Davies (1987) involved the use of pieces of the mantle of *Mytilus edulis*. For clams, pieces of the gonads had to be used to allow us to distinguish clearly between the sexes (pieces of the mantle gave weak unrecognizable colours). Moreover, the boiling time was increased to at least 40 minutes before visual identification. Microscopic analysis of gonad sections confirmed the results obtained with the biochemical technique with an error of 2.5% (n=39) showing that this technique can be used routinely.

Table 1- Sex determination of clams

Dates	October 1999		November 2000	
Sites	Smith's Harbour (n=24)	Little Bay (n=20)	Swift Current (n=20)	Little Bay (n=20)
Sex	7 F, 15 M and 2 NI	7 F, 11 M and 2 NI	11 F and 9 M	15 F and 5 M

F=females, M=males, NI=non identified.

TBARS and catalase assay

The clams collected in 1999 and 2000 were not from the same reference site (Smith's Harbour in 1999, Swift Current in 2000). Sizes were compared (between sites and sampling year) using a one way ANOVA and showed no differences ensuring a homogeneity in clam sampling. No differences between the sexes were found for TBARS or catalase values for both sampling periods.

TBARS values of clams from the two control sites were not different. Catalase values for hepatopancreas from Swift Current(2000) were significantly higher, but gill catalase activities showed no differences (Table 2). A significant difference was found between TBARS values of both hepatopancreas and gills from Little Bay and the reference site in 1999 while no difference was observed in 2000. On the other hand, the catalase activity values showed no differences in the first year of sampling but a significant difference was observed in 2000 between Little Bay and Swift Current clam samples.

A weak but significant negative correlation was found between hepatopancreas TBARS and catalase activity values when combining all data. Due to occurrence of bimodality (catalase) and severe asymetry (TBARS) of the distributions a Spearman Rank Order test for non-normal distributions was used ($p=0.001$, $r=-0.36$).

Table 2- TBARS and catalase activity values in clams

Dates	October 1999				November 2000			
Biomarker	TBARS (nmol/mg)		CATALASE (µmol/min/mg)		TBARS (nmol/mg)		CATALASE (µmol/min/mg)	
Organ	H	G	H	G	H	G	H	G
Reference site	0.237 ± 0.124	0.119 ± 0.067	44.16^{+} ± 13.95	13.60 ± 7.67	0.236 ± 0.163	0.171 ± 0.09	159.90^{+} ± 64.22	8.39 ± 5.11
Little Bay (LB)	0.676* ± 0.446	0.509* ± 0.658	33.09 ± 9.56	16.28 ± 8.30	0.213 ± 0.107	0.144 ± 0.050	202.57* ± 39.61	18.40* ± 13.12
Significant difference	LB > Control site (Smith's Harbour)		No difference		No difference		LB > Control site (Swift Current)	

*significant difference (ANOVA, Tukey test, $p<0.01$), n=20
$^{+}$significant difference between reference sites 1999 and 2000 (T-test, $p<0.05$)
H: hepatopancreas (digestive gland), G: gills

The Comet assay

While preparing digestive gland cells (without trypsin dissociation of tissue) for analysis, the cell viability was found to be 77.3%. The Comet assay was first tested on cells incubated *in vitro* with H_2O_2. This was done in order to validate the experimental conditions. A significant increase in the "length" of DNA was observed in the presence of 600µM H_2O_2 for both hemocytes (Fig.2 and Fig. 3) and digestive gland cells.

No differences between males (n=3) and females (n=5) were found while assessing sex effect DNA damage levels in both hemocytes and digestive gland cells (T-test). To assess variability between individual clams, slides from different animals were analyzed and a variation coefficient was calculated. The average coefficient of variation between individuals was around 30% for both hemocytes and digestive gland cells (n=30 and 38 respectively). Clams from Little Bay and Swift Current (n=8 from each site) were tested for DNA damage levels. No differences in DNA "length" were found between sites for either hemocytes or digestive gland cells.

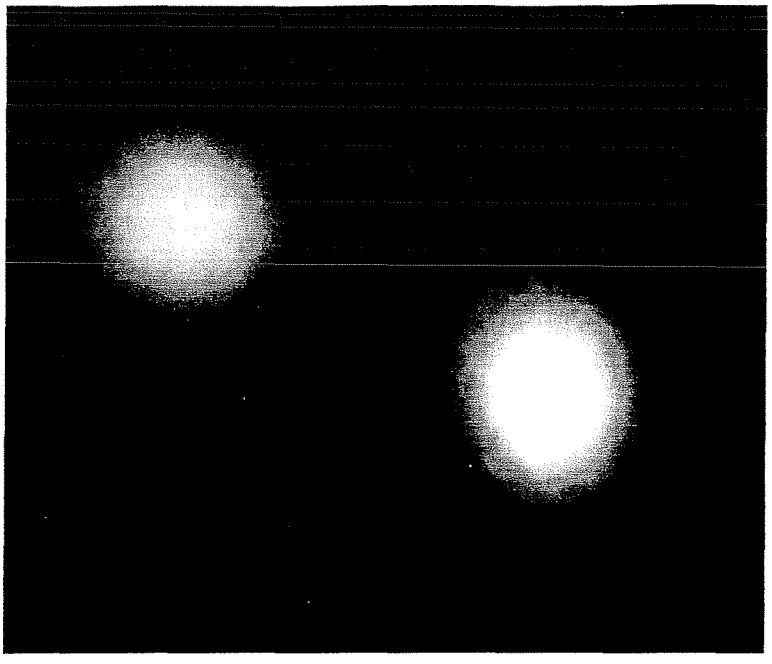

Figure 2- Photomicrograph of two control hemocyte nuclei prepared for Comet analysis (1000X magnification).

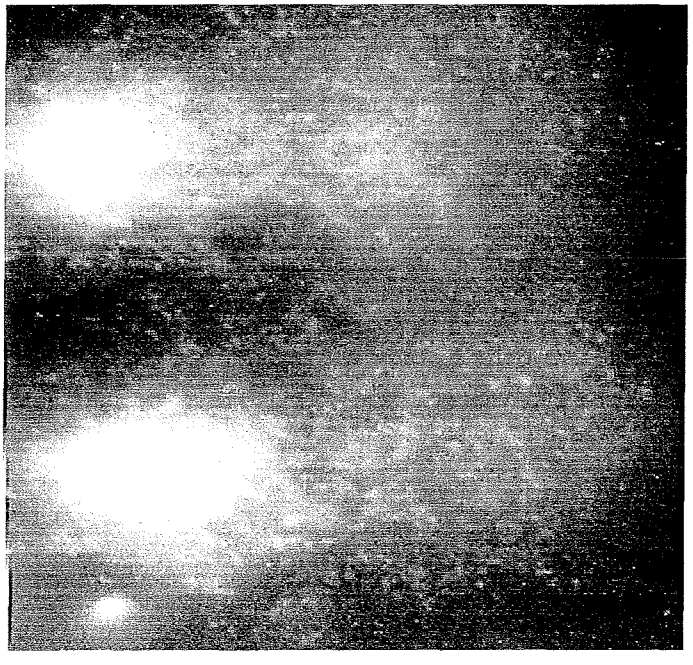

Figure 3- Photomicrograph of hemocytes exposed to H_2O_2 (600 μM) and prepared for Comet analysis (1000X magnification)

Metal analysis
Clams collected in October 1999 were analyzed for Cu (Table 3); a significant difference was found between Little Bay and the reference site (T-test, $p<0.05$). No copper analysis was performed in November 2000.

Table 3- Cu concentration (µg/g DW, dry weight) in *Mya arenaria* soft tissue

Site	Smith's Harbor	Little Bay
Cu concentration	6.99 ± 1.34 (n=5)	62.49* ± 19.05 (n=5)

* significant difference between sites (T-test, $p<0.05$)

Clam histopathology
Detailed examination of the hepatopancreas, gills and gonads of clams living in the tailings delta revealed no evidence of tissue or organ lesions.

Discussion

Soft-shell clams were key sentinel organisms to assess potential biological consequences at the tailings contaminated site. They are facultative deposit feeders and therefore are inclined to take in more from the surface sediment-layer than other bivalve species which are obligate suspension feeders. Thus, the Cu concentration of the sediment may manifest itself in clams but not necessarily in mussels (Hummel *et al.*, 1997). Moreover, geophysical studies of Little Bay revealed that although sulfide-rich material abounds at the mining site, the occurrence of acid mine drainage is not widespread. The low pH typical of acid mine drainage enhances the solubility of metal ions allowing them to become more available (Campbell and Stokes, 1985). The potential toxicity of Little Bay tailing deposits is therefore probably governed by Cu and other metals found in the sediment and the leaching mechanisms operative under near-neutral pH conditions (Kwong and Chaulk, 2000).

Throughout this work, a special effort was made to ensure that there was no effect of sex or size on biomarker responses measured in *Mya arenaria* samples. The analysis of trace metals in another clam species revealed clearly size-related variations for Cu (Colombo *et al.*, 1997). Moreover, many biochemical measurements on *Mytilus edulis* have been found to be sex-dependent (Jabbar and Davies, 1987). The clams collected were between 50 and 80 mm long; the length of clams from different sites were compared and no significant differences were found. No sex effect was observed in TBARS, catalase activity or DNA damage levels in clam specimens thus ensuring the comparability of samples. It is important to point out that a biochemical method traditionally used for sex determination of mussels was used for clams with the exception that we found young gonad tissues to be the tissue of choice for clams. Application of the test to male mussels yields a yellow colour, whereas in females, a pink colour develops (Jabbar and Davies, 1987). For clams, the technique revealed a 97.5%

correspondence between the sex determined after gonad observation and the colour of the reaction product. The colorimetric sexing technique is very useful and can be used routinely in preference to the microscopic examination of 'fixed' sections of the gonads, which is considerably more time consuming.

In the first sampling year (1999), lipid peroxidation (TBARS) levels in both gills and hepatopancreas of clams from the mining site were found to be significantly higher while the activity of catalase, an antioxidant enzyme, was the same in clams from both sites. For the second year (2000), the opposite pathway was observed with no TBARS level differences and significantly higher catalase activities in clams from the mining site. Despite the fact that catalase activities were also different in hepatopancreas samples (but not in gills) when comparing the two reference sites (1999 and 2000), they were still higher when comparing mining and reference site in 2000. Catalase is often one of the earliest enzymes to be induced and also to be highly influenced by seasonal parameters other than pollutants (Cossu *et al.*, 1997). The TBARS difference observed in 1999 could be due to chronic exposure to Cu and other metals like Fe, a metal present in high concentrations in Little Bay (Veinott and Anderson, 2000), coupled to an insufficient level of catalase (and/or other antioxidant pathways). Low lipid peroxidation is the result of effective reactive oxygen species (ROS) scavenging, while higher values reflect either low activity of antioxidative defense or a flux of ROS that exceeds the ROS scavenging capacity of the cell (Angel *et al.*, 1999). A weak but significant negative correlation was found between TBARS and catalase activity values when combining all data. Viarengo *et al.* (1991) found that seasonal variations in antioxidant defense systems in the digestive gland of *Mytilus sp* appeared to be inversely related to the accumulation of lipid peroxidation products in the tissue. Differences in responses between the two years could be explained by the triggering of an antioxidant defense mechanism or directly or indirectly linked to oceanographic differences that could have occurred between October 1999 and November 2000.

The involvement of metals like iron or copper in the increased reactive oxygen species production is intimately related to the level of DNA oxidative damage (Meneghini, 1997). Therefore, we used the Comet assay to determine DNA damage levels in clams during the second year of sampling. While preparing digestive gland cells for analysis, the cell viability was found to be 77.3%. Henderson *et al.* (1998) pointed out that for *in vitro* testing, in order to avoid false positive responses with substances of unknown hazard, cell viability should exceed 75%. Wilson *et al.* (1998) questioned the utility of the Comet assay as an *in vivo* test method for environmental monitoring purposes as the response of mussels treated with H_2O_2 showed high inter-cell variation. The variation coefficient obtained while comparing DNA damage baseline of hemocytes or digestive gland cells from same size range clam specimens was≈30%. In Nacci *et al.* (1996) a similar comparison made between nuclear length averages for individual untreated oysters resulted in a CV of 28%. No differences in DNA damage were found between samples from the mining and reference sites. It may be that euryoxic organisms such as intertidal bivalves have very effective antioxidant defenses to combat major increases in oxyradical production (Livingstone *et al.*, 1990) and thus no consequences can be observed on DNA damage levels.

The copper concentration found in the soft parts of clams collected in Little Bay is significantly higher than in the reference site and is obviously due to contamination by copper ore tailings. Several authors found that Cu concentrations in clam tissues are associated with Cu concentration in the sediment (Cain and Luoma, 1990; Hummel *et al.,* 1997 etc.). The concentration we observed in clams ranged from 6.99 µg/g dry weight (reference site) to 62.49 µg/g dry weight (mining site). Calabrese *et al.* (1984), who conducted a 21 month chronic toxicity study on *Mytilus edulis,* started seeing evident histopathology effects and growth reduction with concentrations between 19.2 and 62 mg/kg fresh weight. On the other hand, the scallop *Mizuhopecten yessoensis* showed a lasting increase in malondialdehyde content in microsomal membranes after Cu exposure when hepatopancreas concentrations were equal to 513 mg/kg dry weight (Chelomin and Belcheva, 1992). It is not unreasonable to assume that copper bioavailability is crucial in determining its potential toxicity (Cohen *et al.*, 1996). Copper can combine with naturally derived organic ligands to form complexes, which are often less toxic and less bioavailable (Adler-Ivanbrook and Breslin, 1999).

An important aspect of our research has been to demonstrate the successful application of the Comet assay to hemocytes and digestive gland cells of soft-shell clams as well as use of a colorimetric technique for determination of clam sex. A difference in lipid peroxidation was found the first year between samples from the mining and reference sites confirming previous results demonstrating that tailings have potential to induce lipid peroxidation (Hamoutene *et al.*, 2000). However, differences were not confirmed in 2000 and no DNA damage was found in clams living in sediments rich in copper ore tailings showing a "controllable" oxyradical production in animal tissues. Moreover, there was no evidence of tissue or organ histopathology which is a valuable measure for assessing health impairment in organisms chronically exposed to toxic substances. Overall, this study as well as our previous results (Meade *et al.*, 2000) which demonstrated no adverse effects on growth in mussels from the tailings site, did not indicate a potential for copper at levels found in Little Bay tailings to pose ecotoxicological risks in the marine environment.

Acknowledgments

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