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Enzyme Cytochemical Responses of Mussels (*Mytilus edulis)* to Resin Acid Constituents of Pulp Mill Effluents

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Effluents and emissions from pulp and paper mills contain toxic constituents that affect water, sediment and air quality and potentially all aquatic biotas (Liss et al. 1997). Past studies have documented effects in fish in association with pulp mill effluents (e.g., Payne et al. 1996 and references therein; Bogdanova and Nikinmaa 1998). A significant percentage of the toxicity in these pollutants is attributable to fatty acids, resin acids and their transformation products such as retene. Resin acids (e.g., pimaric, isopimaric, dehydroabietic and abietic acids) are lipohilic and can bioaccumulate in liver, bile, and plasma of freshwater and estuarine fish and in estuarine clams and amphipods. Rosin, a major product obtained from pine resin, consists primarily of a mixture of abietic- and pimaric-type acids with smaller amounts of neutral compounds (Coppen and Hone 1995).

Histological and histochemical responses of invertebrates, particularly reproductive and digestive cells in bivalve molluscs have been extensively used in monitoring programs on pollution effects as applied in the Mussel Watch Program (reviewed in Bayne et al. 1985; Goldberg 1986). Pulp mill effluents contain large quantities of particulate matter and any effects on bivalves could be indirectly due to reduction in feeding and/or directly due to toxic constituents. Wu and Levings (1980) noted changes in growth rate and reproductive activities in mussels transplanted near a pulp mill effluent in British Columbia. There is interest in using mussels as monitoring species around pulp mills (Salazar 1996) and it is important to determine the potential toxic effects of resin acids.

The morphology and function of digestive diverticula have been described for numerous species including *Mytilus edulis* (Morton 1983). In general, the paired gland consists of many blind-ending tubules that are embedded in vesicular connective tissue matrix and haemal sinuses. The organ is multifunctionally, playing a role in the following functions: (a) intra- and extracellular digestion by the digestive and basophilic cells of the digestive tubule epithelium, respectively (Morton 1983; Robleo and Cajaraville 1997); (b) nutrient storage within the vesicular connective tissue cells (VCT: glycogen) and the adipogranular cells (ADG: glycogen, protein,

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and lipid) (Mathieu and Lubet 1993); (c) wound and cell repair, nutrient digestion and transport, excretion and defense responses by haemocytes (agranular and granular) located in connective tissue and haemal sinuses (Cheng 1981); (d) accumulation of different pollutants and the active participation in detoxification processes. Hydroxylation and detoxification processes of lipohilic xenobiotics in many species are catalyzed by mixed function oxidases (MFOs), involving Glucose-6-Phosphate Dehydrogenase (G-6-PDH) and Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase (NADPH-Diaphorase) (Livingstone 1988, Chayen and Bitensky 1991). It appears that environmental contaminants cause severe changes in the morphology and biotransformation enzyme activities of the digestive diverticula (e.g., Bayne et al. 1985, Cajaraville et al. 1992). In addition, the lipid composition of the gland may change in response to pollutants (Capuzzo and Leavitt 1988).

The objective of the present study was to assess the potential for histochemical and enzyme cytochemical change in the digestive glands of mussels exposed to Rosin, which contains the prototype resin acids found at varying concentrations in pulp mill effluents (Liss et al. 1997). The nominal concentration of Rosin used was approximately 20 ppm. Resin acid levels in effluents may vary from a few ppb to 10,000 ppm or higher, depending on the type of wood, the pulping process, and the nature of water treatment used (Liss et al, 1997). It is also noted that, if resin acids are modified to a minor extent by water treatment, reduction in resin acid levels may not translate into a proportional reduction in toxicity. The overall ecotoxicological significance of resin acid mixtures and associated compounds will depend on the degree of effluent treatment as well as the volume of effluent flows and the degree of dilution in receiving waters.

MATERIALS AND METHODS

Mussels, *Mytilus edulis*, (3.2-4.3 cm length) were collected at a mussel farm (Thimble Bay Farms, Botwood, NF), transferred to tanks with running, air-saturated seawater at a temperature of l-2°C. After an acclimation period of two weeks the animals were divided into two groups of ten animals each:
Control group: The mussels were transferred to a bucket with 22.7 liters aerated seawater for 15 days. The water was replaced three times in this period.
Experimental group: The mussels were exposed to a solution of Rosin (Aldrich Chemical Inc.). Rosin (0.5 gram) was crushed to a very fine powder, transferred to a separatory funnel with 2 liters of saltwater. The solution was shaken vigorously by hand for 10 min. and added to a bucket containing 20.7 liters aerated sea water. The mussels were transferred to this bucket and exposed to Rosin for 15 days. The water was replaced three times with fresh Rosin solution during the experimental period. Mussels of both groups were maintained in unfiltered seawater but were unfed during the two-week experimental period.
After the exposure period one half of each digestive gland was immersed in Histo

Prep (Fisher Diagnostics) in a Peel-a-way embedding mold (Can Lab) before being chilled according to the method of Chayen and Bitensky (1991). The frozen tissue was stored at -70°C until processed for enzyme histochemical study. The other half of each digestive gland was fixed in 10% neutral buffered formalin, dehydrated in ethanol, cleared in Hemo-De (Fisher Diagnostics), impregnated and embedded in paraffin wax (Paraplast Plus, Fisher Diagnostics) for histochemical examination. Consecutive paraffin wax sections (6 µm) of the digestive gland tissue were stained in Masson's trichrome and Periodic Acid Schiff (PAS) with Mayer's hematoxylin and Metanil yellow as counterstains (Kiernan 1990).

Sections (8 µm) of each frozen digestive gland tissue were used for the assessment of lipid storage and enzyme activities. Sudan Black B and Oil red O were used as lysochromes for the determination of all available lipids (other than steroids) and of unsaturated neutral lipids, respectively (Chayen and Bitensky 1991). To demonstrate NADPH-Diaphorase and G-6-PDH activity, sections were incubated with either the substrate reduced nicotinamide adenine dinucleotide phosphate (NADPH) or Glucose-6-Phosphate Disodium Salt.$3H_2O$ (Sigma) and the electron acceptor Nitro blue tetrazolium (Sigma) (Kiernan 1990). Rat liver sections were used for positive controls, while substrate was eliminated from the incubation media in the negative controls. Control incubations were carried out in the presence of the inhibitor *p*-chloromercuribenzoic acid (Sigma) in a concentration of 2.5 mg/mL NADPH-Diaphorase incubation medium. To bypass the diaphorase, the intermediate electron-acceptor phenazine methosulphate (0.15 mg/5.0 mL incubation medium) was added to the G-6-PDH incubation medium immediately before use.

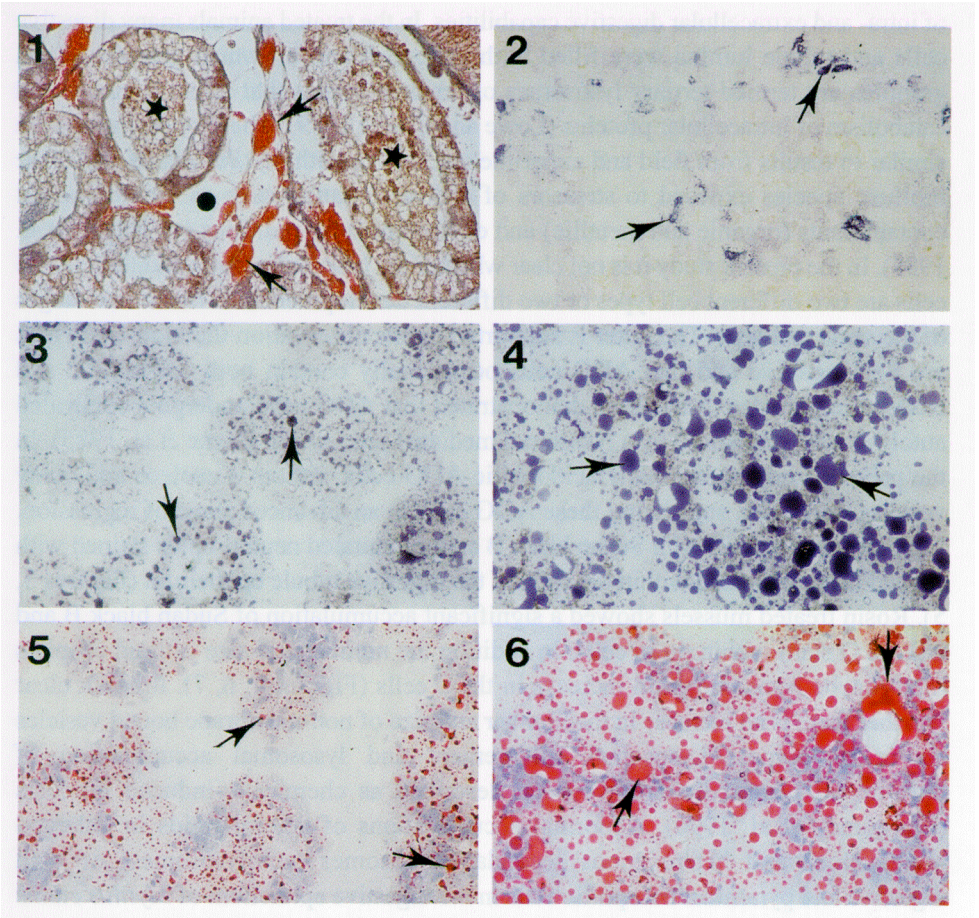
Lipid concentrations (total and unsaturated neutral) and enzyme activities were measured using computerized image analysis. Images were attained from a Zeiss microscope with a Sony video camera attachment and a Bravado image capturing board. Measurements were made with Mocha video analysis software. Concentrations of lipids and enzyme activities are proportional to the number of pixels within intensity thresholds and expressed in arbitrary units of concentration and activity, respectively. Eight readings under low power (total magnification 100x) per digestive gland per animal were carried out on duplicate sections. The results were statistically analyzed by using the interactive statistical analysis program for microcomputers "Statistix 3.1" (Analytical Software, St. Paul, MN). Significant differences between test groups were determined by one way ANOVA.

RESULTS AND DISCUSSION

Histological examination of the digestive diverticula showed, that Rosin caused degeneration of the digestive cells (increased vacuolization, accumulation of residual bodies, fragmentation and shedding), an increase in basophilia in the granulocytes and the occurrence of and vacuolization in multinuclear acidophilic adipogranular cells (Figure 1). Degeneration of the digestive epithelium may result in a gradual loss

of intra- and extracellular digestive capabilities. In the treated animals many digestive cells and tubule lumina were filled with yellowish and brownish granules. Such granules are termed tertiary lysosomes or residual bodies that affect turnover and catabolism of intracellular proteins (Lowe and Clarke 1989). These observations are similar to results from field and experimental studies with *Mytilus edulis* and other mollusc species exposed to stressors of a diverse nature such as environmental contaminants (organic and metallic) and environmental conditions (cf. Bayne et al. 1985). In the present study it is not clear whether the basophilic and acidophilic ADG cells are two different cell types or two different developmental or functional stages of the same cell type. The latter is supported by the observation that both cell types are PAS positive and that ADG cells occasionally contain both acidophilic and basophilic granules. It has been demonstrated that ADG cells undergo a controlled autolysis (degranulation) by a lysosomal mediated process (cf. Bayne et al. 1985). In the present study occasionally acidophilic ADG cells without a nucleus and many vesicles were seen, suggesting these ADG cells in an advanced stage of regression. Lipid droplets stained with Sudan black B and unsaturated neutral lipids stained with Oil red O were located within the cells of the digestive tubule epithelium (Figures 3, 5). Rosin treated mussels showed a significant accumulation of Sudan black B and Oil red O stain within these cells. In addition the number and size of lipid droplets (and maybe lysosomes) did increase in these cells (Figures 4, 6, 7). It is not clear whether there is an increase in the number and size of non-membrane bound vesicles and/or lysosomes. Lysosomal enlargement and lysosomal accumulation of unsaturated neutral lipids have been described as chemically-induced lipidosis (Margómez et al. 1990). Abnormal accumulations of neutral lipids in enlarged homogenous and heterogenous secondary lysosomes (autolysosomes) of both digestive and pyramidal basophilic cells in the digestive epithelium of *Mytilus edulis* exposed to a mixture of petroleum hydrocarbons and copper were observed (Lowe and Clark 1989). However, the question of whether contaminants related abnormal accumulation of lipids is a result of an increase of lipid synthesis, or an inability to catabolize lipids, or an impairment in the control of lipid storage remains unanswered (Lowe and Clark 1989). Responses of *Mytilus edulis* to lipophilic contaminants (aromatic hydrocarbons and/or polychlorinated biphenyls) in field and mesocosm studies resulted in elevations in lipid content and 1ipid:protein ratios of digestive glands and differential changes in both neutral lipid (triacylglycerols) and phospholipid pools, altering both adaptive and energetic responses (Capuzzo and Leavitt 1988).

Sites of NADPH-Diaphorase activity are epithelial cells lining the ducts, granulocytes, and ADG cells within the connective tissue around the ducts and between the digestive tubules. The sites of G-6-PDH activity are similar to those of NADPH-Diaphorase. The overall enzyme activities within the connective tissue between the digestive tubuli were significantly increased in Rosin treated mussels compared to activity in the controls (Figures 2, 8). These enzymes are linked with the



Cross section of the digestive gland of *Mytilus edulis*. Magnification: x225.

Figure 1. Histological section of a Rosin treated mussel, showing vesicular cells (●), numerous acidophilic adipogranular cells (arrows), and degenerating digestive tubules (★). Masson's Trichrome.

Figure 2. Frozen section of a Rosin treated mussel, showing strong NADPH-diaphorase activity in granulocytes and adipogranular cells (arrows).

Figure 3. Frozen section of a control mussel, showing fine lipid droplets within the cells of the digestive epithelium (arrows). Sudan black B.

Figure 4. Frozen section of a Rosin treated mussel, showing an accumulation of lipids within the cells of the digestive epithelium (arrows). Sudan black B.

Figure 5. Frozen section of a control mussel, showing small unsaturated neutral lipid droplets within the cells of the digestive epithelium (arrows). Oil red O with Hematoxylin counterstain.

Figure 6. Frozen section of a Rosin treated mussel, showing an accumulation of unsaturated neutral lipids within the cells of the digestive epithelium (arrows). Oil red O with Hematoxylin counterstain.

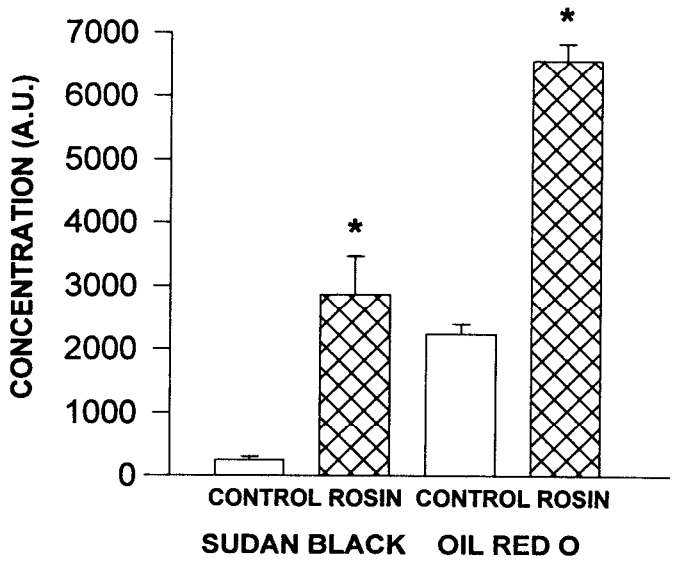


Figure 7. The effect of Rosin on the total lipid (Sudan black B) and unsaturated neutral lipid storage (Oil red O) in the digestive glands of mussels. Each bar represents a mean ± sem; *=significantly different from the control: P=0.0001 and 0.0000, respectively.

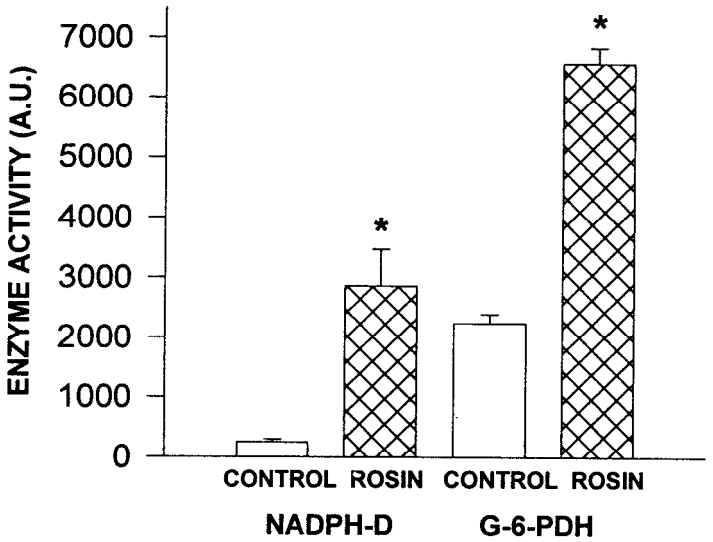


Figure 8. The effect of Rosin on NADPH-D and G-6-PDH activity in the granulocytes and adipogranular cells in the digestive glands of mussels. Each bar represents **a** mean ± sem;*= significantly different from the control: P=0.0001and 0.0000, respectively.

Phase I - (oxidative transformation) xenobiotic detoxification through the Mixed Function Oxidase (MFO) or cytochrome P-450 mono-oxygenase system. The site of action of both Phase I- and Phase II (conjugation) enzymes of xenobiotic detoxification may be the smooth endoplasmic reticulum (SER) and/or the cytoplasm and mitochondria. In *Mytilus edulis* this system is primarily located in the SER of the digestive gland (Livingstone 1988). It has been demonstrated that in the blood cells and/or digestive cells of mussels *(Mytilus edulis* and *Mytilus galloprovincialis)* activities of enzymes of the phase I metabolism (MFO system: e.g., NADPH-ferrihemoprotein reductase or NADPH-cytochrome c [P-450 or neotetrazolium reductase]) and phase II metabolism of xenobiotics are enhanced after exposure to different organic pollutants in field and experimental studies (Bayne et al. 1985; Cajaraville et al. 1992). Bleached Kraft Pulp Mill effluents did induce strongly hepatic cytochrome P-450-dependent enzyme 7-ethoxyresorufin-*O*-deethylase activity in perch, *Perca fluviatilis* (Balk et al. 1993). Bucher et al. (1993) did observe an induction of hepatic aryl hydrocarbon hydroxylase (component of MFO-system) combined with weakened antioxidant defense (e.g., reduction of G-6-PDH activity) in the liver of bullheads, *Cottus gobio* L., exposed to treated paper mill effluents, suggesting an enhanced risk of oxidative damage. Specific G-6-PDH activity in the blood cells of *Mytilus edulis* increased in response to aromatic hydrocarbons (Bayne et al. 1985).

From the present histopathological and enzyme cytochemical study it is concluded that Rosin is potentially toxic to mussels around pulp mill sites in a marine environment and it indicates the importance of assessing the relative health of bivalve populations in the vicinity of pulp and paper mills. It also supports use of mussels as a biological monitoring species around the same.

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