Lipid class and fatty acid composition of mussel, *Mytilus trossulus*, in Vancouver Harbour

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Introduction

Lipids are divided broadly into two categories: namely, neutral lipid (NL), which is the stored fat and is mainly composed of triglycerides, and phospholipids (PL) and cholesterol, which are building blocks of membranes. Identification of lipid composition is important for physiological studies. Furthermore, PCBs and other oragano-chlorine contaminants are known to accumulate in tissue, and the information for lipid composition is helpful to explain the mechanism for the accumulation of these chemicals.

Fatty acids are the principal components in lipids. Their diversity in terms of chain length, degree of unsaturation, geometry, and position of the double bonds is responsible for the definitive characteristics of lipids for different organisms (Gutnikov 1995).

The Iatroscan TLC method was used to separate lipids by thin layer chromatography using the hydrogen flame ionization detector (FID). This method was developed by Okumura *et al.* (1975). The next step was done with an adsorbent sintered thin layer chromatographic quartz rod that consisted of silica gel powder fused by fine glass powder as the binding agent and an automatic scanner, which contains a hydrogen-FID for sample detection. The combination of these two steps makes quantitative TLC a rapid and easy method for the routine analysis of lipids separated by regular TLC.

The PICES Practical Workshop was held from May 24 to June 7, 1999, in Vancouver Harbour, Canada. In this study, the lipid and fatty acid composition of mussel, *Mytilus trossulus,* were determined at 7 sites (I-1, I-2, I-3A, I-4, I-5B, I-6, and I-7) in Vancouver Harbour (Fig. 1).

Methods

One hundred mussels of various sizes were gathered at each site. Mussels were shelled, and soft tissues were homogenized. Lipids were extracted from 5 g subsamples using a 30 ml solvent mixture of chloroform-methanol (2:1, v/v) (Blig and Dyer 1959). The chloroform layer, (which contains dissolved lipids) was collected, washed with 0.88% potassium chloride, and removed completely using a rotary-evaporator and a centrifugal-evaporator. Then, the concentration of lipids was adjusted to 100 mg/ml with chloroform. The separation of NL and PL was performed using a Sep-pak Silica washed with 10 ml chloroform. 50 µl of chloroform with extracted lipids was then loaded onto the Sep-pak. The NL was eluted with 8 ml chloroform, and the PL was eluted with 10 ml methanol.

For the determination of the lipidic composition, 0.2 µl of chloroform containing the extracted lipids was spotted onto the base of Chromarods, and developed with hexane-diethylether-acetic acid (70:30:1). After the solution was developed to a certain position, Chromarods were dried at 100ºC, and lipids were analyzed by an Iatroscan. Fatty acids in NL and PL were analyzed according to the methods of the American Oil Chemists' Society (A.O.C.S.) (1991). The extracted lipids were removed using nitrogen gas and a centrifugal evaporator, and saponificated with 0.5 N sodium hydroxide at 100ºC for 5 minutes. Then, the saponificated samples were methylated by 14%

boron trifloride methanol complex methanol solution at 100ºC for 30 minutes. After the methylation, fatty acids were dissolved in Isooctane, and analyzed by GC/MS.

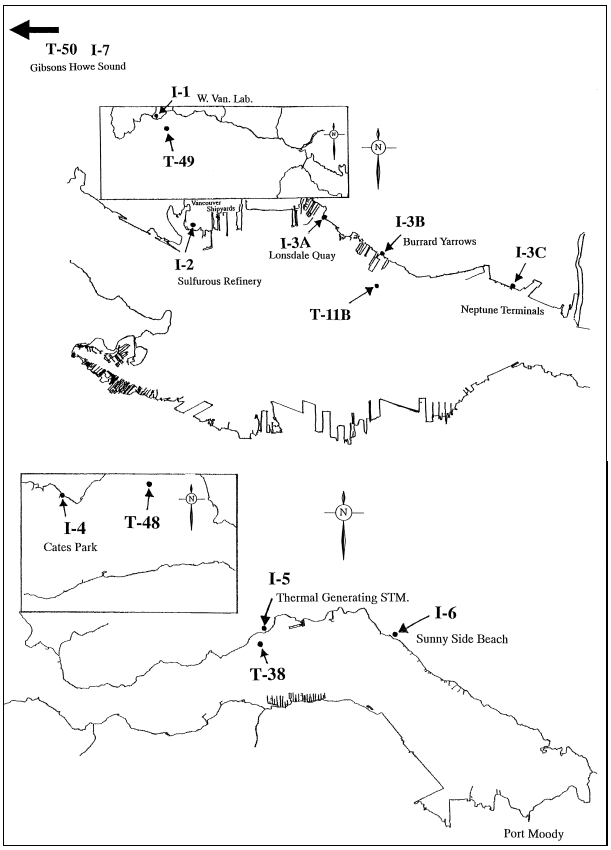
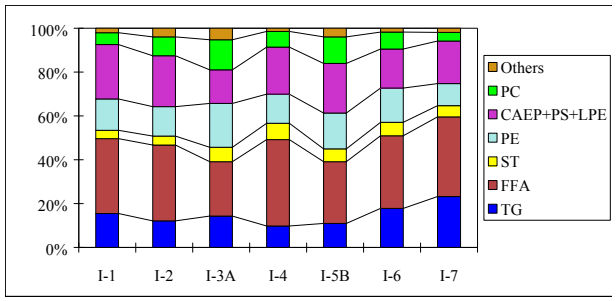


Fig. 1 Sampling sites in Vancouver Harbour.

Results and discussion

Lipid composition in mussels

Results of analysis by the Iatroscan TLC showed that the main lipids in mussels were triglyceride (TG), free fatty acid (FFA), sterol (ST), and phospholipid (PL). The ratios of these compounds to total lipid were 10 - 23% for TG, 24 - 37% for FFA, 4 - 7% for ST, and 36 - 55% for PL (Fig. 2). Furthermore, we analyzed PL using a thin layer chromatograph (TLC) and the Iatroscan TLC. TLC results showed that phospholipid was composed of phosphatidylethanolamine (PE), ceramide 2-aminoethyl phosphate (CAEP), phosphatidylserine (PS), lysophosphatidyl-



TG, Triglyceride; FFA, Free Fatty Acid; ST, Sterol;
PE, Phosphatidylethanolamine;
CAEP, Ceramide 2-aminoethylphosphonate;
LPE, Lysophosphatidylethanolamine;
PS, phosphatidylserine;
PC, Phosphatidylcholine;
Others, Lysophosphatidylcholine+Unknown component

Fig. 2 Lipid composition in *M. trossulus* (weight % to total lipid).

ethanolamine (LPE), phosphat-idylcholine (PC), lysophosphatidyl-choline (LPC), and others. The individual quantities of CAEP, PS, and LPE could not be determined, because they were not separated completely. The ratios of compositions in the PL were 27 - 37% for PE, 28 - 55% for CAEP+PS+LPE, 11 - 25% for LPC. All components of total lipids are shown in Figure 2.

The depot lipid is mainly TG and the lipid composition changes depending on the nutrient condition. On the other hand, the tissue lipid is mainly PL and its composition does not change. On the basis of these lipidic characteristics, we tried to evaluate the nutrient conditions of mussels at all sampling sites using the TG/PL ratio (Fig. 3). The ratio was highest at site I-7 and the nutrient condition of mussels at this site appeared to be better than at other sites.

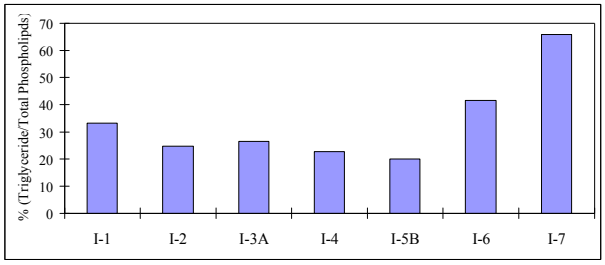


Fig. 3 Tryglyceride to total phospholipids ratio in *M. trossulus*.

It is well known that the oxidation and hydrolysis of lipids in fish and shellfish during frozen storage cause serious deterioration of quality (e.g. Jeong, *et al*. 1990; Shimada and Ogura 1990; Refsgaard *et al*. 1998). Jeong *et al*. (1990) reported that the contents of TG and PC in oyster, *Crassostrea gigas*, decreased during storage at –20°C while the concentration of free fatty acids increased. In this study, the mussel samples were stored at –20°C until analysis. Samples were transported from Canada to Japan on dry ice. Under these circumstances, in our analysis the content of FFA in total lipid could be higher, and content of TG and PC could be lower, than those in live animals. But all samples were kept in the same condition until analysis so that the nutrient condition among the sampling sites might be compared from the TG/PL ratio.

Ota *et al*. (1990) reported that TG was the main component of total lipid (TL) in rainbow trout (91.3%) and other fishes. According to Ozawa *et al*. (1993), the TG content in TL of kokanee salmon's muscle was 65.3% for the dorsal portion, 82.8% for the ventral portion, and 66.7% for the tail. Kawasaki *et al*. (1994) also reported that the TG content in TL in firefly squid's liver was 60.9 - 72.4%. Since the quantity of TG changes considerably with the season, the comparison between mussel and other aquatic animals is difficult. But the TG concentration in mussel was lower than that in fish and squid.

Fatty acid composition

The fatty acid composition was identified for 41 classes by GC/MS. Table 1 shows the fatty acid composition in lipids for those classes that were more than 1% of the total fatty acids. The dominant components of total fatty acids were 16:0, 16:1n-7, 18:1n-7, 20:5n-3, and 22:6n-3. The composition of fatty acids in NL was 41 classes, while that in the PL was 29 classes. Especially, 20:5n-3 and 22:6n-3 contained higher levels of fatty acids and they were 8.8 - 18.8% and 6.4 - 14.5%, respectively. The compositions of 20:4, 20:5, 22:5, and 22:6 are special for aquatic organisms (Koike and Tsuchiya 1988), and these ratios were 20 - 33% in total fatty acids.

Similar levels of 14:0, 16:0, 16:2n-7, 18:0, and 20:5n-3 were found in NL and PL. The contents of 17:0, 18:1n-9, 18:1n-7, 18:3n-3, 18:4n-3, and 20:2n-6 were higher in NL than those in the PL. On the other hand, the contents of 16:1n-7, 20:5n-3, 22:1n-11, and 22:6n-3 were higher in PL than in NL.

Jeng et al. (1990) reported that the percentages of polyenoic acid in PL, NL, and TL for *C. gigas* decreased and the percentage of saturated acids increased during storage at –20°C. In this study, unsaturated fatty acids could be underestimated in comparison with lipid compositions found in live animals.

Conclusion

In this study, the lipid and fatty acid composition in mussel, *M. trossulus*, was determined at Vancouver Harbour, Canada, during the PICES Practical Workshop. The main components of lipid were tryglyceride, free fatty acid, sterol, and phospholipids, which were composed of phosphatidylethanolamine, ceramide 2-amino-ethylphosphonate, phosphatidylserine, lyphosphat-idylethanolamine, phosphatidylcholine, and lysosphatidylcholine. The ratio of triglyceride in total lipid was lower than that in fish.

The dominant components of total fatty acids were 16:0, 16:1n-7, 18:1n-7, 20:5n-3 and 22:6n-3. The composition of fatty acids in neutral lipid and phospholipid was identified for 41 and 29 classes, respectively.

Generally, the fatty acid composition is influenced by feeding, season, water temperature, and depth of the habitat (Hori and Itasaka 1978). In the future, if the plant and animal plankton of food for mussels can be gathered at the sampling sites, the dietary life could be estimated from the fatty acid composition.

The fatty acid and lipid composition might have deteriorated during transportation from Canada to Japan, and during storage until analysis. If more accurate quantitative analysis is needed, lipid determinations will have to be performed as soon as the samples have been gathered.

Table 1. Fatty Acid Components in Total , Neutral, and Phospholipids of *M. trossulus*.

	I-1			I-2			I-3A			I-4			I-5B			I-6			I-7		
Fatty Acid	**TL**	**NL**	**PL**	**TL**	**NL**	**PL**	**TL**	**NL**	**PL**	**TL**	**NL**	**PL**	**TL**	**NL**	**PL**	**TL**	**NL**	**PL**	**TL**	**NL**	**PL**
14:0	3.6	3.7	2.9	2.4	2.4	2.5	1.9	2.0	1.6	3.0	3.2	2.8	3.4	3.5	3.1	2.9	2.8	2.9	5.4	5.7	4.4
16:0	18.9	19.3	15.6	16.0	15.4	17.5	15.9	16.5	15.0	15.5	15.3	15.7	15.7	16.0	15.1	15.9	15.8	16.2	14.9	15.3	13.5
16:1n-7	5.0	4.2	11.2	8.5	8.3	9.2	8.4	9.6	6.4	6.5	4.4	9.4	6.5	3.7	11.3	6.7	5.7	10.3	10.6	9.2	16.0
16:2n-7	1.9	2.2	1.6	1.0	1.4	1.6	0.8	1.2	1.6	0.8	1.3	1.5	1.0	1.5	1.2	1.0	1.2	1.5	1.5	1.9	1.4
17:0	1.9	1.9	-	1.5	1.5	-	1.5	1.5	-	1.4	1.4	-	1.3	1.3	-	1.5	1.5	-	1.6	1.6	-
18:0	2.8	2.8	2.8	2.8	2.7	3.1	3.1	3.0	3.1	2.6	2.5	2.8	2.5	2.4	2.7	2.7	2.7	2.7	2.2	2.2	2.3
18:1n-9	3.1	3.2	2.0	2.3	2.9	0.6	2.6	3.2	1.5	2.3	2.8	1.8	3.0	3.5	2.0	2.7	3.0	1.9	2.6	2.9	1.5
18:1n-7	5.9	6.2	3.7	4.3	4.7	3.3	4.8	5.8	3.1	4.6	5.3	3.6	4.8	5.4	3.9	5.1	5.4	3.7	6.2	6.9	3.4
18:2n-6	2.2	2.3	1.3	1.4	1.6	1.0	1.6	1.9	1.0	2.1	2.5	1.6	2.6	3.1	1.7	2.4	2.6	1.8	2.0	2.2	1.2
18:3n-3	3.0	3.3	-	1.9	2.1	-	1.3	2.0	-	2.5	3.1	-	3.4	3.4	-	2.2	2.9	-	1.6	1.6	-
18:4n-3	7.1	7.7	3.0	4.3	5.0	2.6	3.7	4.7	1.9	5.1	6.6	3.0	4.8	7.6	3.3	5.3	6.1	2.4	1.4	1.8	1.8
20:1n-11	0.8	0.7	1.7	1.0	0.9	1.4	1.5	1.6	1.3	1.1	0.9	1.3	1.1	0.9	1.5	1.2	0.9	2.1	0.9	0.7	1.5
20:1n-9	1.5	1.2	3.1	4.2	4.7	3.0	5.2	6.1	3.6	3.3	3.4	3.3	3.5	3.8	2.9	4.3	4.7	3.0	3.3	3.6	2.3
20:1n-7	4.2	4.4	2.2	3.0	3.3	2.3	2.6	2.8	2.4	3.1	3.5	2.6	3.0	3.4	2.3	3.5	3.8	2.4	2.9	3.2	1.8
20:2*	3.4	3.5	2.7	1.7	1.3	2.9	3.2	2.4	4.7	1.9	1.2	3.0	1.9	1.2	3.1	1.7	1.1	3.8	1.5	0.8	3.9
20:2*	1.1	1.1	1.2	0.7	0.7	0.9	1.2	0.9	1.6	1.0	0.9	1.2	0.7	0.7	0.8	0.9	0.8	1.0	0.9	0.7	1.5
20:2n-6	1.2	1.2	0.8	1.0	1.0	0.7	0.9	1.0	0.7	1.1	1.2	0.9	1.3	1.4	1.2	1.2	1.3	1.0	0.7	0.8	0.5
20:4n-6	2.0	2.1	1.5	1.9	2.0	1.6	3.4	3.8	2.6	2.0	2.1	1.8	1.7	1.8	1.4	2.4	2.6	1.7	2.5	2.7	2.1
20:5n-3	8.8	7.2	20.7	15.3	13.0	21.4	11.0	5.4	20.9	17.6	16.9	18.5	16.8	15.1	19.8	14.5	13.6	17.7	18.8	18.6	19.5

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CYP1A and related measurements in English sole (*P. vetulus*) from Vancouver Harbour

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Introduction

Vancouver Harbour is a busy seaport and gets a considerable influx of anthropogenic pollutants including metals, PCBs, organochlorine pesticides, and polyaromatic hydrocarbons. English sole (*Pleuronectes vetulus*) are bottom-feeding fish and subject to bioaccumulation of lipophilic hydrocarbon compounds, which contaminate the sediments in the harbour and are linked to toxicity in various marine organisms. English sole is an excellent sentinel species for monitoring marine ecosystem health because they are relatively slow growing, are widely distributed throughout the harbour and adjacent waters, and individual fish have a small home range or forage in a relatively confined area. In addition, English sole is a potential source of human contaminant exposure because it is fished commercially.

Induction of hepatic microsomal cytochrome P450 (CYP) enzymes is a common and characteristic biochemical response to halogenated hydrocarbon exposure that accompanies and often precedes toxicity in all animals examined thus far. CYP is a large and ubiquitous group of hemeproteins found in fish, mammals, birds, plants, and microorganisms that catalyze the oxidative biotransformation of diverse lipophilic xenobiotic and endogenous compounds. Because CYP

enzymes play a critical role in the metabolism, bioaccumulation, and potential toxicity of halogenated and nonhalogenated hydrocarbons found in the food chain, levels of individual CYP enzymes are important determinants of susceptibility to environmental contaminant exposure. CYP enzyme induction in fish populations has been suggested as a sensitive biochemical marker of contaminant exposure, and by inference, of marine ecosystem health (Safe 1990; Goksøyr and Förlin 1992; Stegeman *et al*. 1992; Addison 1996; Addison *et al*. 1994; Campbell *et al*. 1996). Induction of the CYP1A subfamily of enzymes can be determined by measurement of associated enzymes activities such as ethoxyreosufin O-deethylase (EROD) and benzo[a]pyrene hydroxylase or by measuring CYP1A protein using immunochemical methods.

The purpose of the present study was to measure EROD activity and CYP1A protein levels in liver tissue of English sole from five sites in and around Vancouver Harbour, and to compare these biochemical parameters with sediment levels of hydrocarbon pollutants measured at these same sites.

Materials and methods

Liver samples

English sole were collected by trawl net during May and June 1999, from five sites in and around Vancouver Harbour. The sites were designated as T-50 (Howe Sound), T-49 (West Vancouver), T-11B (Lonsdale Quay), T-38 (Port Moody), and T-48 (Indian Arm) (see Section I, Fig. 1.4). Thirty fish were collected from each site. Fish were weighed, separated by sex, and a blood sample was taken. Fish were then killed by dissection of the spinal cord, and livers were removed and placed into ice-cold Tris-HCl buffer, pH 7.4. Hepatic microsomes were prepared from 68 male and female fish (at least 10 fish per site), by differential centrifugation. Microsomal pellets were suspended in 0.25 M sucrose and aliquots of the suspension were stored at –75°C until used.

Twenty additional English sole were collected from site T-49 for use as positive and negative controls for the microsomal CYP assays in a controlled exposure experiment. These fish were housed in salt-water aquaria at a temperature of 8°C in the West Vancouver Facility. After acclimation for 5 days, the fish were weighed and 10 fish in one aquarium tank were treated with β-naphthoflavone (β-NF) in corn oil by a single i.p injection at a dosage of 50 mg/kg. Ten fish in a second tank were treated similarly with corn oil (vehicle) only at a dosage of 0.25 ml/100 g body weight. One week after treatment, the fish were killed by dissection of the spinal cord, weighed, and liver microsomes were prepared as described above.

Determination of cytochrome P450 and protein

Total CYP content was determined from the carbon monoxide difference spectrum using the method of Omura and Sato (1964). Protein concentration was measured by the method of Lowry *et al.* (1981).

Enzyme assays

Microsomal EROD activity was measured using a spectrofluorometric assay as described by Burke *et al.*(1985). Each microsomal sample was assayed directly in a fluorescence cuvette incubated at room temperature (22–25°C) using a Shimadzu Model RF-540 fluorometer interfaced with a Shimadzu DR-3 data recorder.

Preparation of antibodies

Antibody against CYP1A was raised in female New Zealand rabbits immunized with a synthetic peptide corresponding to trout CYP1A coupled to keyhole limpet hemocyanin as described previously (Lin *et al*. 1998). This antibody is specific for mammalian CYP1A1 and recognizes a single CYP1A protein in all fish species tested to date.

Immunoblots and densitometric quantitation

Polyacrylamide gel electrophoresis (PAGE) was performed essentially as described by Laemmli (1970). English sole liver microsomal samples were applied to gels at a final concentration of either 2 or 5 pmol total microsomal CYP per lane. Microsomal proteins resolved on SDS-PAGE were

transferred electrophoretically to nitrocellulose and probed with antibodies as described by Towbin *et al.* (1979). Blots were incubated with anti-cytochrome P450 1A peptide IgG at a concentration of 10 μg IgG/ml. Bound primary antibody was located using alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody. Immunoreactive proteins were detected by reaction with a substrate solution containing 0.01% NBT, 0.05% BCIP, and 0.5 mM $MgCl_2$ in 0.1 M Tris-HCl buffer, pH 9.5. Assay conditions were optimized to ensure that colour development did not proceed beyond the linear response range of the phosphatase reaction. Staining intensities of the bands were quantified with a pdi 420 oe scanning densitometer connected to an IBM-type personal computer using Quantity One® Version 3.0 software (pdi Inc., Huntington Station, NY). The amount of immunoreactive protein was determined from the integral of the optical density of the stained band. Staining intensities of bands on each blot were normalized with a purified rat hepatic CYP1A1 standard that was included on every gel as an internal standard.

Statistical analysis

Data are presented as the mean ± standard error of the mean of values determined from 10-20 fish per trawl site. Correlations between hepatic microsomal EROD activities and CYP1A protein levels were analyzed by simple linear regression. Coefficients of variation (r^2) with a p value <0.05 were considered statistically significant.

Results

Table 1 lists mean values of body and liver weight, and total cytochrome P450 (CYP) content for liver microsomes prepared from fish treated with corn oil and β-naphthoflavone. As can be seen from the data, liver weight was decreased and the total CYP content was increased for fish treated with β-naphthoflavone in comparison with corn oil-treated fish. Table 2 lists mean values of total CYP content for liver microsomes prepared from fish collected from 5 sites. As can be seen from the data, the mean value of total CYP content was variable for fish collected from the different sites.

EROD activity was measured in English sole liver microsomes. Mean values of fish from the five sites, along with mean values of the β-naphthoflavone and corn oil-treated fish, are shown in Figure 1. Treatment with β-naphthoflavone resulted in a large increase in EROD activity (approximately 18-fold) compared with corn oil-treatment. EROD activity was also elevated in fish from sites T-50 and T-38 compared to fish from sites T-49 and T-11B. In fact, the mean EROD activity of fish from site T-50 was approximately 11-fold greater than the mean EROD activity of corn oil-treated fish and 4-fold greater than that of fish from site T-49.

English sole liver microsomes were analyzed on immunoblots probed with antibody generated to a synthetic peptide corresponding to trout CYP1A1. This antibody detected a single protein band in the microsomal preparations, implying that English sole liver contains one protein that is immunochemically related to trout CYP1A1. As seen on the immunoblot in Figure 2, the CYP1A band in microsomal preparations of fish from site T-50 was stained more intensely than the band in fish from site T-49, indicating that there is increased expression of CYP1A protein in fish from site T-50 relative to site T-49.

Table 1. English sole treated with corn oil or β-naphthoflavone.

Parameter	**Corn oil**	**b-NF**
Number of fish	10	10
Age (yr)	n.d.	n.d.
Body weight (g)	132.3 ± 12.3	103.8 ± 14.4
Liver weight (g)	1.49 ± 0.13	0.97 ± 0.10*
Total CYP content (nmol/mg protein)	0.29 ± 0.02	0.54 ± 0.05*
Number of female fish	5	5/6
Number of male fish	5	5/4

Values for body weight, liver weight, and total CYP content are expressed as the mean ± SEM.

* Indicates that the value is significantly different from that of the corn oil-treated group.

Table 2. English sole from five sites in Vancouver Harbour.

Site	Number of fish	Mean age (yr)	Total CYP content (nmol/mg)	Number of male fish	Number of female fish
T-50 (Howe Sound)	14	7.7	0.46 ± 0.03[a,b]	5	9
T-49 (West Vancouver)	20	6.2	0.29 ± 0.02[a]	13	6
T-11B (Lonsdale Quay)	10	7.5	0.31 ± 0.04[b]	4	6
T-38 (Port Moody)	12	10.5	0.37 ± 0.02	2	10
T-48 (Indian Arm)	12	8.1	0.38 ± 0.03	4	8

Values for total CYP content are expressed as the mean ± SEM.
[a] indicates that the value is significantly different for these two groups ($p < 0.001$).
[b] indicates that the value is significantly different for these two groups ($p < 0.05$).

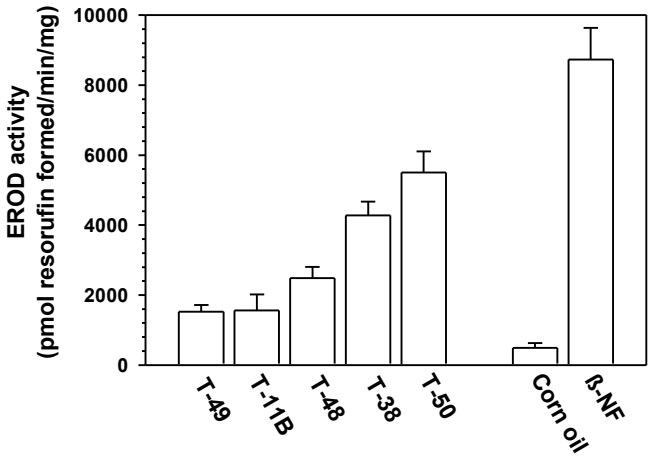

Fig. 1 Hepatic microsomal EROD activity of English sole from Vancouver Harbour.

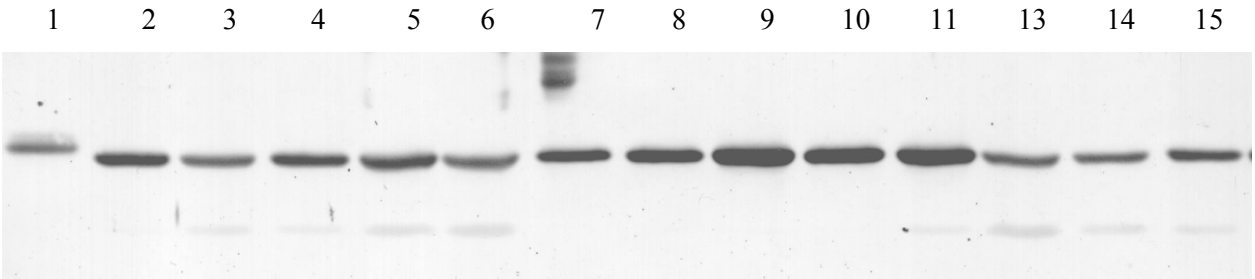

Fig. 2 Representative immunoblot of hepatic microsomes from English sole probed with anti-CYP1A peptide IgG. Samples were applied to the gel at the concentrations indicated. Lane 1 contains purified rat CYP1A1 (1.0 pmol/lane), lane 2 contains liver microsomes from a fish from site T-48 (2 pmol/lane), lanes 3-6 contain liver microsomes from individual fish from site T-49 (5 pmol/lane), lanes 7-11 contain liver microsomes from individual fish from site T-50 (2 pmol/lane), and lanes 13-15 contain liver microsomes from individual fish from site T-49 (5 pmol/lane).

The microsomal CYP1A protein in all 88 English sole was quantified by densitometry and the data are displayed in Figure 3. As was the case with EROD activity, the mean CYP1A protein level was increased after treatment with β-naphtho-flavone (approximately 13-fold) relative to fish treated with corn oil. CYP1A protein levels were elevated in fish from sites T-50, T-38, and T-48 compared to fish from sites T-49 and T-11B. The CYP1A expression livers of fish from site T-50 was approximately equal to that of β-naphtho-flavone-treated fish and was 7-fold greater than that of fish from site T-49.

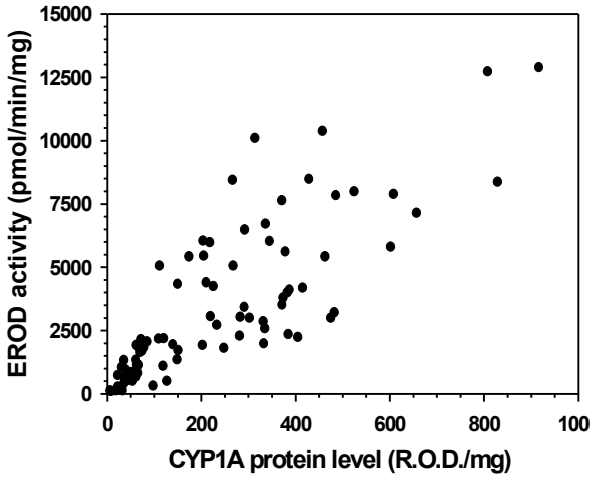

Fig. 3 Hepatic microsomal CYP1A protein levels in English sole from Vancouver Harbour.

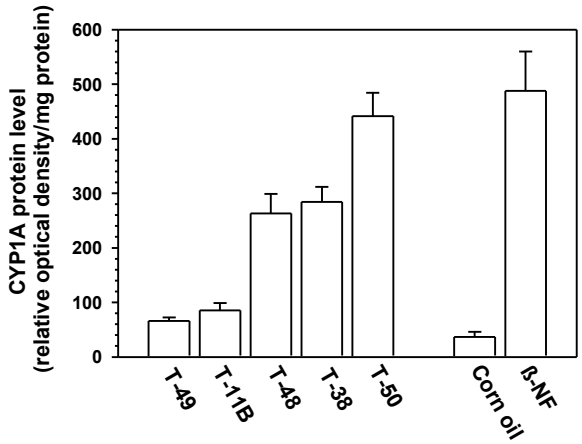

Fig. 4 Correlation between hepatic EROD activity and CYP1A protein levels in English sole samples.

The relationship between CYP1A protein levels and EROD activity for all 88 fish was examined (see Fig. 4). As expected, CYP1A protein levels were found to be highly correlated with EROD activity ($r^2 = 0.66$, $p < 0.05$).

No correlation was found between age of the English sole and CYP1A levels or EROD activity. When fish were segregated according to sex, no correlation was found between sex and EROD activity for fish from most of the sites. The exception was site T-11B, where EROD activity was greater in female than male fish, but the number of male and female fish in this group, as in most of the other groups, was too small for rigorous statistical analysis.

The relationship between CYP1A levels and sediment concentrations of various organochlorine and polyaromatic hydrocarbon compounds was examined. Sediment chemistry data from the PICES Vancouver Harbour Workshop indicated high levels of high molecular weight aromatic compounds (>4000 ng/g dry weight) and high levels of PCBs (>40 ng/g dry weight) at site T-48, with slightly lower levels at site T-38 (4500 and 34 ng/g dry weight, respectively), and even lower levels at site T-49 (2000 and 9.5 ng/g dry weight, respectively). Site T-50 is unusual in that very low or undetectable levels of these compounds were found at this site. Thus, it appears that, except for site T-50, there is a positive correlation between the amount of CYP1A in English sole and total aromatic hydrocarbon and total PCB levels in sediments at these sites.

Discussion

The present study, using English sole liver samples collected from 5 sites in and around Vancouver Harbour demonstrated the following:

1. Hepatic microsomal EROD activity was induced 18-fold by β-naphthoflavone treatment and was 5 to 9 times greater in fish from sites T-38 (Indian Arm), T-48 (Port Moody), and T-50 (Howe Sound) than in corn oil-treated fish.
2. Hepatic CYP1A protein levels were 5 to 6 times greater in fish from sites T-38 (Indian

Arm), T-48 (Port Moody), and T-50 (Howe Sound) than in corn oil-treated fish.

3. Hepatic microsomal EROD activity and CYP1A protein levels were well correlated, supporting the role of CYP1A as the primary catalyst of EROD activity in English sole.
4. A comparison of sediment chemistry data showed that fish with increased CYP1A expression came from sites (T-38 and T-48) containing relatively high levels of polyaromatic hydrocarbon (PAH) and organochlorine compounds, suggesting that CYP1A was induced in English sole by environmental exposure to PAHs and PCBs and related compounds.
5. The sediment data does not explain the high EROD activity and CYP1A protein levels found in fish from site T-50 (Howe Sound). We assume that induction of CYP1A induced in these fish was caused by environmental exposure to effluent from pulp and paper mills nearby (e.g. the Port Mellon mill).
6. Hepatic EROD activity and CYP1A protein levels in English sole are effective indicators of hydrocarbon pollutant levels in the marine environment.

Acknowledgements

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Contamination of organotin compounds and imposex in molluscs from Vancouver, Canada

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Introduction

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPhT), have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s, and have caused imposex in neogastropods and mesogastropods in the world (Goldberg 1986; Horiguchi 2000). Imposex is defined as a superimposition of male sexual organs (penis and vas deferens) on female gastropods, and may bring about reproductive failure at severely affected stages (Smith 1971; Gibbs and Bryan 1986; Gibbs *et al*. 1987, 1988, 1990). Imposex is thought to be endocrine disruption induced by TBT and TPhT in gastropods (Matthiessen and Gibbs 1998).

The use of TBT has been banned in antifouling paints for ships smaller than 25 m in length in many developed countries, such as European countries and the United States, since the 1980s (Stewart 1996). In Japan, the production, import and use of organotins (TBT and TPhT) have been regulated by law and administrative guidance since 1990, resulting in no production in 1997 (Horiguchi 2000). TBT-based antifouling paints, however, have still been used in developing countries, such as Asian countries, and also for most vessels larger than 25 m in length (Stewart 1996; Horiguchi 2000). The worldwide ban of TBT is being discussed by the Marine Environmental Protection Committee (MEPC) of the International Maritime Organization (IMO) (Horiguchi, 2000).

A PICES Practical Workshop was held from May 24 to June 7, 1999, in Vancouver, Canada. The aim of this study is to know the tissue concentrations of organotin (butyltin and phenyltin) compounds in molluscs (gastropods and bivalves) from Vancouver, the imposex symptoms in gastropods around Vancouver, and to assess the present status on organotin contamination in Vancouver.

Materials and methods

Molluscan specimens (gastropods and bivalves) were collected at 15 sites near Vancouver and Victoria during the Workshop. After sampling them, raw or frozen gastropod specimens were used for imposex identification: sex determination and imposex identification were anatomically done (Gibbs *et al*. 1987). The degree of imposex was expressed as incidence (frequency) (%), Relative Penis Length (RPL) Index (%), Relative Penis Size (RPS) Index (%) and Vas Deferens Sequence (VDS) Index through the measurement of penis length and observation of the development of vas deferens (Gibbs *et al*. 1987; Horiguchi *et al*. 1994).

Chemical analysis of organotin (butyltin and phenyltin) compounds in tissues of both gastropod and bivalve specimens were conducted by the methods described in Horiguchi *et al*. (1994). Briefly, tissues were extracted with 0.1% tropolone/benzene and 1N HBr/ethanol by ultrasonication, derivatized with propylmagnesium bromide, cleaned by silica gel column chromatography and quantified by gas chromatography with a flame photometric detection (GC-FPD). The detection limit of the instrument was 50 pg, and certified reference material of Japanese sea bass, *Lateolabrax japonicus*, for TBT and TPhT analysis (prepared by the National Institute for Environmental Studies; NIES CRM No. 11) was used for quality

assurance and quality control. The analytical conditions are described in more detail in Horiguchi *et al*. (1994).

Results and discussion

No neogastropod specimens (e.g. *Nucella lima*) were collected at sites in Vancouver in this study. No neogastropod specimens were collected either in the survey around Vancouver in 1994 (Tester *et al*. 1996). Neogastropods, such as *Nucella*, however, were observed around Vancouver in the 1970s (Levings, personal communication). It is possible that neogastropod populations have been wiped out by some biological and/or environmental factors in Vancouver since the 1980s.

Results on imposex survey in the file dogwinkle, *Nucella lima*, and the frilled dogwinkle, *Nucella lamellosa*, from Ogden Point, Clover Point and Ten-mile Point in Victoria, and from Mission Point in Wilson Creek (see Section I, Fig. 1.5) are shown in Table 1. Slightly affected imposex was observed in populations of both the file dogwinkle and frilled dogwinkle (3.3 - 19.0, 0.004 - 0.7 and 1.1 - 2.9 for RPL, RPS and VDS Indices in the file dogwinkle and 8.2 – 23.1, 0.1 – 1.2 and 1.0 for RPL, RPS and VDS Indices in the frilled dogwinkle, respectively) although the incidences of imposex were high (71-100% and 100% in populations of the file dogwinkle and the frilled dogwinkle, respectively).

Butyltin concentrations in tissue of both the file dogwinkle and frilled dogwinkle are shown in Figure 1. Phenyltin compounds were not detected in both the file dogwinkle and frilled dogwinkle. Regarding TBT, 2.4 – 14.4 ng/g wet wt. and 6.5 – 22.0 ng/g wet wt. were detected in the file dogwinkle and frilled dogwinkle, respectively. Total butyltin concentrations in tissue (sum of TBT and its metabolites, monobutyltin (MBT) and dibutyltin (DBT)) of the file dogwinkle and frilled dogwinkle were 7.3 – 28.8 ng/g wet wt. and 10.8 – 44.0 ng/g wet wt., respectively.

Table 1. Imposex in the File Dogwinkle (*Nucella lima*) and the Frilled Dogwinkle (*Nucella lamellosa*) from Victoria (Ogden Pt., Clover Pt. and Ten-Mile Pt.) and Wilson Creek (Mission Pt.).

Imposex in the File Dogwinkle (*Nucella lima*)			
	Ogden Pt.	**Clover Pt.**	**Ten-Mile Pt.**
Frequency(%)	100	72	71
RPL Index (%)	19.0	11.8	3.3
RPS Index (%)	0.7	0.2	0.004
VDS Index (%)	2.9	2.1	1.1

Imposex in the Frilled Dogwinkle (*Nucella lamellosa*)		
	Ten-Mile Pt.	**Mission Pt.**
Frequency(%)	100	100
RPL Index (%)	8.2	23.1
RPS Index (%)	0.1	1.2
VDS Index (%)	1.0	1.0

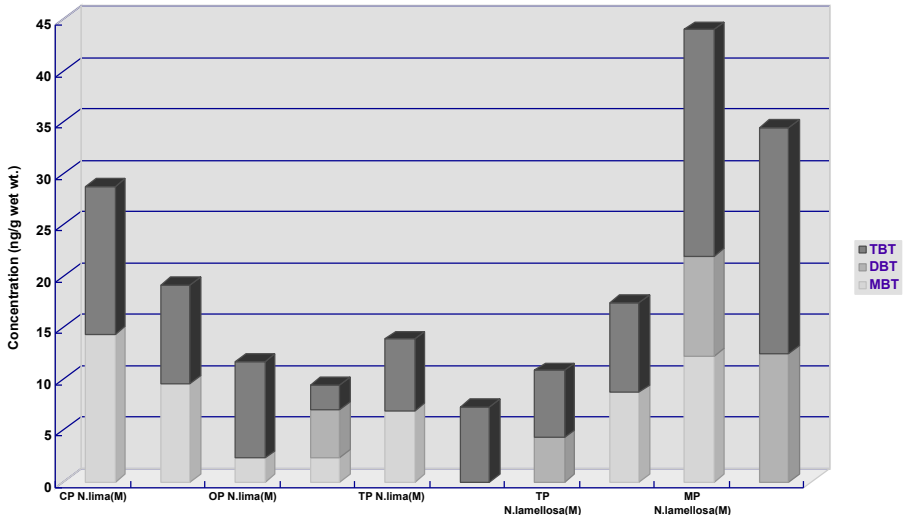

Fig. 1 Tissue concentrations of butyltin in the dogwinkle.

Comparison of these analytical values with reported concentrations of TBT and/or butyltin compounds in tissues of organisms shows that TBT and/or butyltin concentrations detected in the dogwinkles from the sites of Victoria and Wilson Creek were relatively low (Belfroid *et al*. 2000; Environmental Agency of Japan 1999; Tanabe *et al*. 1998; Takahashi *et al*. 1997). As imposex seems to have been extensively caused by relatively low contamination levels of TBT in dogwinkle populations surveyed in this study, it is suggested that dogwinkles may be sensitive to TBT and that imposex may be induced even at a low environmental concentration of TBT in dogwinkles. Under laboratory experimental conditions, imposex was induced at 64 ng/l of average exposure concentration of TBT for 120 days in the file dogwinkle, and bioconcentration factor of TBT was estimated to be approximately 2200 (Stickle *et al*. 1990).

Biological monitoring using the foolish mussel, *Mytilus trossulus*, was also carried out to determine the present status on organotin contamination in Vancouver. Results on chemical analysis of organotin compounds in tissues of the foolish mussel specimens are shown in Figure 2. Phenyltin compounds were not detected in the foolish mussel specimens either. Butyltin compounds were detected in foolish mussel specimens from all of sites surveyed, including a reference site (I-7), with a maximum concentration of 173.2 ng/g wet wt. (I-4). TBT was the most predominant among butyltin compounds detected in the foolish mussel, except for the specimens from I-3-A station: DBT was the most predominant among butyltin species detected in the foolish mussel from I-3-A, possibly suggesting some sources of the contamination of DBT near I-3-A because DBT has been used in PVC stabilizer.

Concentrations of TBT detected in tissues of the foolish mussel from Vancouver in this study were relatively high, compared with those of TBT in marine organisms reported in recent publications, although they were below the tolerable average residue level of Canada (Belfroid *et al*. 2000; Environmental Agency of Japan 1999; Takahashi *et al*. 1997). TBT concentrations in sediment core samples collected from Vancouver Harbour

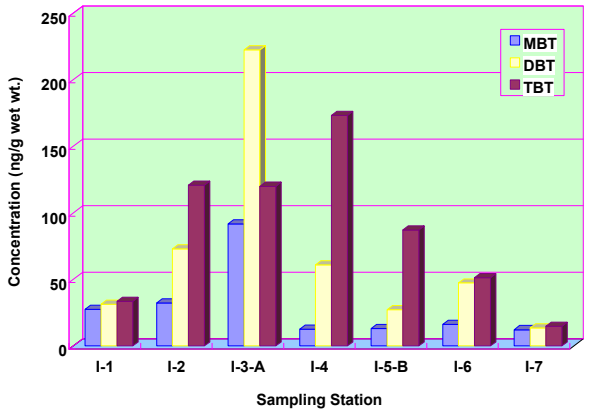

Fig. 2 Tissue concentrations of butyltins in the foolish mussel from Vancouver Harbour (May-June 1999).

(Burrard Inlet) do not show the temporal declining but still high (Thompson 1997). It could result from a continuous use of TBT in antifouling paints for vessels larger than 25 m in length, and a persistence of TBT in bottom sediments. TBT contamination was therefore confirmed to have still continued in Vancouver. Based on the results mentioned above, it is strongly believed that one of the causal factors having wiped out neogastropod populations in Vancouver is TBT from antifouling paints.

A remarkable difference of TBT accumulation in tissue was observed among the bivalve species (Fig. 3). The highest concentration of TBT was detected in the horse clam, *Tresus capax* (2229.9 ng/g wet wt.). Although bioconcentration factor of TBT and/or bioavailability of TBT through contaminated sediment are unknown in the horse clam, remarkably high concentration of TBT in tissue may have caused some adverse effects in the horse clam because some chronic toxicities have been observed in bivalves by exposure to low concentrations of TBT (Alzieu and Heral 1984; Thain and Waldock 1986; Bryan *et al*. 1987; Lawler and Aldrich 1987; Salazar and Champ 1988). Further study is necessary to examine possible adverse effects in the horse clam. Regarding the ratio of butyltin species in tissue, TBT was the most predominant in almost all bivalve specimens surveyed, suggesting low metabolic rate of TBT in these bivalve species. Phenyltin compounds were not detected in bivalves other than the foolish mussel either.

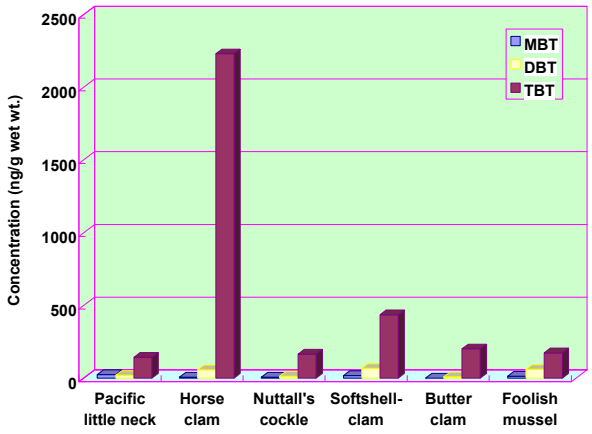

Fig. 3 Tissue concentrations of butyltins in bivalves at station I-4 in Vancouver Harbour (May 30, 1999).

The highest TBT concentration in tissue was consistently observed in the Pacific oyster, *Crassostrea gigas*, among the marine invertebrates collected in every intertidal zone of 3 sites of Japan (Horiguchi et al., unpublished data). As TBT concentrations in tissue were also consistently higher in the Pacific oyster than in the foolish mussel in this study, the Pacific oyster could be useful for biological monitoring of TBT contamination.

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