Bull. Environ. Contam. Toxicol. (1996) 56:121-128

Bulletin of Environmental Contamination and Toxicology

Distribution and Elimination of [^{14}C] in Saithe (*Pollachius virens* L.) after Application of a Single Dose of [^{14}C] Polyhexamethylene Hydrochloridebiguanide

R. Christiansen, K. H. Palmork

Institute of Marine Research, Division of Environmental Research, Postbox 1870, N-5024 Bergen, Norway

Received: 26 January 1995/Accepted: 12 June 1995

Biguanides were early shown to have antibacterial activity (Curd and Rose 1946). The antibacterial activity of polybiguanides was later shown to be superior to that of the monomeric biguanides (Davies and Field 1969), and polymeric biguanides like polyhexamethylene biguanides (PHMB) were developed. Polymeric biguanides have general applications as disinfectants, antiseptics and preservatives. Vantocil IB (I.C.I. Ltd.), a poly dispersed mixture of PHMBs, is an environmental biocide. This chemical has been used as a bactericide in concentration of 100 ppm by the Oil industry in the North Sea for treating new gas pipelines in order to prevent corrosion caused by sulfate reducing bacteria. After treatment the waste water is discharged into the sea. More recent uses of PHMB are in the treatment of the eye infection keratitis, which is caused by an amoebae (Larkin et al. 1992) and within various ophthalmic products (Dexter et al. 1989).

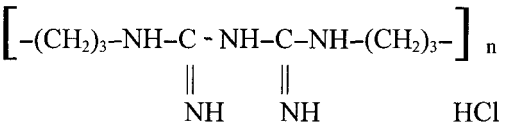
Few studies have been done on the bioaccumulation of PHMB, and the knowledge of bioaccumulation of the biocide in aquatic organisms is poor. Bratt and Hathway (1976) studied the bioaccumulation and excretion of [^{14}C] in rats fed [^{14}C]-labeled PHMB. The acute oral LD50 dose of the PHMB Vantocil IB (I.C.I. Ltd.) for rats has been determined to be at least 4.0 g kg^{-1} body weight (Boardman 1969). The aim of the present study was to determine the distribution and elimination of biguanide and its metabolites in different tissues of fish after oral injection of a single dose of [1^{4}C]-labeled PHMB and to evaluate the importance of bile and urine as excretion routes for the component.

MATERIALS AND METHODS

Saithe (*Pollachius virens* L.) (mean weight 302 g), caught in the coastal waters near Bergen, Norway, were used in the experiment. Before dosing, the fish were acclimated for 1 wk without feeding. The fish were kept in a 2000-L tank supplied with seawater at a flow rate of 20 L min^{-1}.

Correspondence to: R. Christiansen

Poly(biguanide-1,5-diyl [1,6 - ^{14}C]hexamethylenehydrochloride) (95% purity) was purchased from Amersham International Ltd. (Amersham, U.K.). The general formula of PHMB is:



with n values ranging from 2 to 40 with a mean of 5.5. The compound was prepared as described in Bratt and Hathway (1976) with n ranging from 1 to 5 (molecular weight: 180-900). The tissue solubilizer Soluene-350 and the liquid scintillation cocktails Dimilume-30 and Insta-gel were obtained from Packard Instruments (Illinois, USA). All other reagents used were of analytical grade. The radioactive labeled PHMB had a specific activity of 7.1 µCi mg^{-1} and a purity of 95 % determined by thin-layer chromatography at the manufacturing laboratory. PHMB was dissolved in distilled water to a concentration of 12.5 mg mL^{-1}, equivalent to 88.8 µCi mL^{-1}. Gelatin capsules were half-filled with a commercial salmon diet, to which 25 µL of the PHMB solution was added. This gave a dose of 2.22 µCi in each capsule or approximately 0.67 µCi kg^{-1} fish. The capsules were introduced to the stomach of the fish using a modified 5 mL plastic syringe affixed to a plastic tube holding the capsule, as described by Solbakken et al. (1979).

After dosing the fish were divided in groups of five to six and placed into nine 260-L tanks supplied with running water (10 L min^{-1}) for experimental periods lasting less than 48 hr. The fish which were sampled during the period from 96-1032 hr, were kept in a 2000-L tank supplied with seawater at a flow rate of 20 L min^{-1}. The salinity was kept at 34 % and the temperature ranged from 7 to 9°C. Every second to third day, the fish in the 2000-L tank were fed thawed sprat (*Spratus spratus* L.). At time intervals of 14, 18, 22, 24, 26, 28, 30, 32, 36, 48, 96, 192, 384 and 1032 hr, the of fish were sacrificed by a blow to the head, and stored at -20°C until dissected. At the time of the sampling, the length and weight of the fish were measured and the condition factors were calculated as follows: (100 x W/ L^3) (Bagenal and Tesch 1978), where W is the wet weight of the fish and L the fork length of the fish. The liver somatic index (LSI) were calculated as percentage liver weight of total weight.

Gall bladder with bile, urinary bladder with urine and samples from muscle and liver tissues were analyzed for radioactivity. The gall and urinary bladder were dissected while the fish were half frozen to minimize the lose of urine and bile. Three parallel muscle samples, approximately 0.1 g, were dissected from the white trunk muscle of each fish between the first dorsal fin and the lateral line. The liver was removed from the body cavity and rinsed in methanol to remove external [^{14}C]. The wet weight was recorded before dissecting three samples of approximately 0.1 g from the liver lobes. All the samples were placed in scintillation vials, and the weight recorded. Tissue solubilizer was added and the samples were placed in an incubator at 50 °C overnight for digestion. Isopropanol

and hydrogen peroxide were used to bleach gall bladder and liver samples. After digestion and bleaching the scintillation cocktail was added to the vials. The activity of [^{14}C] in different tissues and body fluids of the saithe was measured using a Packard 300 CD liquid scintillation counter (Packard Instrument Co., Downers Grove, USA), equipped with an automatic quenching correction system. The activity is measured as disintegrations min^{-1} (dpm). The counting time was set to 5 min. The background activity was measured in vials containing only the scintillation cocktail and the tissue solubilizer. The background activity was subtracted from all the measured values before the calculations were done.

The total amount of radioactivity in the muscle and liver was calculated from the measured dpm values. The muscle comprises approximately 50 % of the total weight of saithe and the percentage [^{14}C] in the muscle was calculated using this value. The amount of [^{14}C] in the different tissues was expressed as percentage of the a plied dose. In urine and bile the activity was expressed as disintegrations $min^{-1}g^{-1}$ (dpm g^{-1}).

The data were subjected to regression analyses and one way analyses of variance (ANOVA) and difference between groups determined at the 5 % level by the Student-Newman-Kuels multiple ranges test. All statistical tests were done using the statistical package CSS: Statistica (Complete Statistical Systems, Tulsa, USA).

RESULTS AND DISCUSSION

The fish were observed to eat in all groups that were offered food. The mean weight, condition factor, liver weight and LSI of the fish at the different samplings are given in Table 1.

Table 1. Exposure time, number of fish per group, body weight and length, condition factor and liver somatic index (LSI) of saithe (*Pollachius virens* L.) fed radioactive labelled PHMB (mean ± SEM). Numbers with different letters are significantly different.

Time (hr)	Number of fish	Weight (g)	Condition factor	Liver weight (g)	LSI
0	5	302 (19)^{ab}	0.75 (0.03)^{a}	7.4 (0.38)^{a}	2.14 (0.38)^{a}
14	5	312 (12)^{ab}	0.75 (0.02)^{a}	11.3 (0.64)^{a}	3.62 (0.64)^{ab}
18	5	288 (19)^{ab}	0.80 (0.02)^{ab}	11.0 (0.46)^{a}	3.89 (0.46)^{b}
22	5	305 (12)^{ab}	0.74 (0.02)^{a}	13.0 (0.96)^{a}	4.37 (0.96)^{bc}
24	5	311 (6)^{ab}	0.80 (0.04)^{ab}	9.0 (0.28)^{a}	2.89 (0.30)^{ab}
26	4	300 (17)^{ab}	0.76 (0.02)^{a}	8.8 (0.37)^{a}	2.95 (0.50)^{ab}
28	6	292 (10)^{ab}	0.84 (0.03)^{bc}	10.4 (0.62)^{a}	3.60 (0.62)^{ab}
30	6	299 (10)^{ab}	0.80 (0.02)^{ab}	10.2 (0.41)^{a}	3.35 (0.41)^{ab}
32	6	304 (21)^{ab}	0.78 (0.04)^{ab}	7.5 (0.47)^{a}	2.52 (0.48)^{ab}
36	6	279 (12)^{a}	0.79 (0.02)^{ab}	11.1 (0.84)^{a}	4.04 (0.84)^{b}
48	4	301 (20)^{ab}	0.78 (0.03)^{ab}	9.2 (0.40)^{a}	2.96 (0.40)^{ab}
96	5	305 (13)^{ab}	0.85 (0.04)^{bc}	11.0 (0.60)^{a}	3.61 (0.60)^{ab}
192	5	331 (8)^{b}	0.80 (0.03)^{ab}	10.6 (0.28)^{a}	3.21 (0.28)^{ab}
384	5	314 (18)^{ab}	0.81 (0.03)^{ab}	11.5 (0.24)^{a}	3.65 (0.24)^{ab}
1032	6	417 (36)^{c}	0.92 (0.04)^{c}	25.8 (0.92)^{b}	5.80 (0.92)^{c}

The one way ANOVA showed that the weight, condition factor, liver weight and LSI of the fish in the different groups were affected by time. A slight statistical increase was observed in the fish weight ($Y = 295.68 + 1.1 \cdot 10^{-1}X$, $r = 0.60$), the condition factor ($Y = 0.78 + 1.3 \cdot 10^{-4}X$, $r = 0.44$), the liver weight ($Y = 9.23 + 1.4 \cdot 10^{-2}X$, $r = 0.62$) and the LSI ($Y = 3.20 + 2.4 \cdot 10^{-3}X$, $r = 0.42$) from the start to the end of the experiment. Y is the fish weight, condition factor, liver weight and LSI respectively and X the time in hr. The effect of dilution of the [^{14}C] concentration in the tissue caused by growth was avoided by expressing the accumulation of [^{14}C] as percentage of the administered dose. The exposure of the fish to [^{14}C]-labeled PHMB did not cause any observable morphological changes in the fish and mortality was not observed during the experimental period.

The accumulation and elimination pattern of radioactivity in different tissues and body fluids varied. The distribution of radioactivity in the muscle, liver and gallbladder of the fish at different time intervals following the oral ingestion of [^{14}C] is shown in Table 2.

Table 2. Distribution of radioactivity in muscle, liver, urine and gallbladder in saithe (*Pollachius virens* L.) at different time intervals following an oral dose of PHMB. The amount of [^{14}C] in muscle and liver are given as percentage [^{14}C] of the given dose and the amount of [^{14}C] in urine and gallbladder as disintegrations $min^{-1}g^{-1}$ (mean ± SEM). Values with different letters are significantly different ($p<0.05$).

Time (hr)	Number of fish	Muscle %	Liver %	Urine dpm g^{-1}	Bile dpm g^{-1}
14	5	3.5 (0.31)^{a}	16.6 (1.28)^{a}	56355 (13487)^{a}	21116 (12650)^{a}
18	5	2.3 (0.42)^{ab}	7.2 (1.17)^{bc}	51924 (10854)^{a}	16157 (6060)^{a}
22	5	2.6 (0.26)^{ab}	6.4 (1.55)^{bc}	66021 (8517)^{a}	9562 (1904)^{a}
24	5	2.0 (0.24)^{ab}	11.5 (1.65)^{c}	49764 (5815)^{a}	11713 (4143)^{a}
26	4	2.6 (0.08)^{ab}	8.2 (1.93)^{bc}	43950 (12338)^{a}	15100 (2700)^{a}
28	6	2.1 (0.24)^{ab}	9.1 (1.48)^{bc}	45534 (10369)^{a}	17880 (5866)^{a}
30	6	3.0 (0.51)^{ac}	9.8 (1.06)^{bc}	46479 (10479)^{a}	14161 (3020)^{a}
32	6	2.9 (0.45)^{ac}	7.7 (1.40)^{bc}	57391 (6390)^{a}	30935 (16668)^{a}
36	6	2.2 (0.16)^{ab}	10.6 (1.31)^{c}	49407 (9867)^{a}	11410 (4021)^{a}
48	4	2.1 (0.47)^{ab}	7.0 (0.98)^{bc}	32093 (10613)^{ab}	13754 (27507)^{a}
96	5	2.3 (0.32)^{ab}	5.2 (1.26)^{b}	36861 (9026)^{a}	4723 (1704)^{a}
192	5	1.1 (0.24)^{b}	2.1 (0.69)^{d}	5397 (1910)^{b}	2032 (597)^{a}
384	5	1.6 (0.29)^{bc}	1.6 (0.19)^{d}	2826 (578)^{b}	1360 (328)^{a}
1032	6	1.4 (0.20)^{b}	0.3 (0.03)^{d}	677 (381)^{b}	286 (85)^{a}

When determining the distribution and elimination of a compound using radioactive labeled molecules, the concentration of the radioactive atom is measured. Further metabolism of the compound can not be determined using a scintillation counting method only. The activity measured would be [^{14}C] from PHMB, metabolites of PHMB or fragments of PHMB incorporated in biological molecules. Larger molecules usually have a low potential to bioaccumulation due to steric hindrance at passage through cell membranes and substances with a

molecular weight >700 is unlikely to bioaccumulate (Anon 1994). About 63.5 % of the PHMB administrated had molecular weights above 700 and possibly have to be broken down in the intestine prior to absorption. The major part of the compound had a molecular weight of 720 (58.4 %).

A small amount of the applied dose accumulated in the muscle and the variation in the analyzed fish from the same groups was large. The concentrations of [^{14}C] in the muscle ranged from 3.5 % at the first sampling to 1.4 % at the last sampling. The uptake of [^{14}C]-labeled PHMB was rapid, and the highest levels of activity were measured in the samples from the earliest experimental period. There was no significant difference between the groups sampled between 14 to 96 hr. There was, however, a significant difference in concentration between the group sampled at 14 and the groups sampled at 192 to 1032 hr ($p<0.05$) (Table 2).

An increased level of activity was found in the liver as compared with the muscle. The concentrations of [^{14}C] in the liver decreased from 16.6 % 14 hr after the application to 0.3 % at the last sampling at day 43 after the application. As for the muscle, the uptake of [^{14}C]-labeled PHMB was rapid, and the highest levels of activity were measured in samples from the earliest experimental period. The one way ANOVA showed that the activity in liver was significantly affected by time. The concentration of [^{14}C] in the fish at the first sampling was significantly higher than the concentration in the fish at the later samplings. There were no significant differences in concentration from 18 to 96 hr. The concentrations in the liver were, however, significantly lower at the samplings taken at 192 to 1032 hr. The amount of radioactivity found in the liver during the first 96 hr was 3 to 5 times higher than the values recorded for the muscle tissue. The depletion of [^{14}C] in the liver with time was more pronounced than in muscle. The concentration in the liver of the fish was lower than the concentration found in the muscle at the sampling after 1032 hr.

The level of radioactivity found in the liver and muscle showed that only a part of the applied [^{14}C] from PHMB was absorbed and accumulated. This may be due, in part, to the high water solubility of the compound (octanol/water coefficient of $2.39 \cdot 10^{-3}$). Individual differences in food uptake, water intake and gastric emptying time might also lead to variations in the amount of PHMB absorbed. About 63.5 % of the PHMB had a molecular weight above 700 and the molecules will probably have to be broken down prior to absorption in the gastro-intestinal tract, however, a large part of the compound had a molecular weight of 720. The digestibility of the compound for fish is not known. The accumulation of xenobiotics in organisms is often expressed as the bioconcentration factor (BCF), which is the ratio between the concentration of the chemical in the water and the tissue measured at equilibrium. The BCF is related to the octanol/water partition coefficient, which is the ratio at the equilibrium concentration of the chemical between water and octanol. There is a linear relationship between log BCF and log (octanol/water partition coefficient) (Neely et al. 1974; Veith et al. 1979). PHMB is highly soluble in water and the bioaccumulation should be expected to be low.

Saithe given [9-^{14}C]-phenanthrene absorbed most of the given dose and 72 % of the administered dose was found in the liver shortly after exposure, whereas only 6 % of the dose was found in the muscle. The elimination from the liver was higher than from muscle (Solbakken et al. 1979). The present results show a much lower accumulation of radioactivity in fish fed [^{14}C]-PHMB. The mean value of radioactivity in the period from 14 to 48 hr is 9.4 % of the given dose in liver and 2.5 % in the muscle. The values found in the bile and the urine were low. The elimination of radioactivity from the muscle was very slow, and at 43 days, 40 % of the total accumulated [^{14}C] was still left. The elimination of [^{14}C] from the liver was much higher and at 43 days only 1.8 % of the total accumulated dose was left. The metabolic activity in the liver is high, producing energy and building blocks for biosynthesis. The liver is also an important organ in the detoxification process of xenobiotics in the organism.

Roubal et al. (1977) found a higher accumulation of [^{14}C] anthracene than [^{14}C] naphtalene in rainbow trout. The highest concentration was found in the gall bladder. The values for the liver were higher than the values found for the muscle. The most water soluble compound, naphthalene, was eliminated faster than anthracene. Accumulation of [^{14}C] tetrachlorobiphenyl in rainbow trout was found to be higher in the muscle than in the liver, and the rate of elimination was low (Guiney et al. 1977). The rate of elimination of hexachlorbiphenyl has also been found to be low in flounder (Solbakken et al. 1984). In general, the lipophilic compounds show a much higher degree of accumulation than less lipophilic compounds and increasing persistence with increasing lipophilicity. The accumulation of [^{14}C] in fish fed [^{14}C] PHMB is low and a large part of this xenobiotic is probably eliminated via the feces as was observed in rats who eliminated 93 % of a single dose of PHMB (Bratt and Hathway 1976).

The excretion of [^{14}C] in the urine and bile is expressed as dpm g^{-1} (Table 2). The activity in the gallbladder was on an average 3.5 times lower than in the urine. There were no statistical differences in activity in the gallbladder at the different samplings, although large variations were found in the activity within the same group. The concentration of [^{14}C] in the gallbladder, however, was only 1-2 % of the concentration found in liver. The one way ANOVA showed a significant decrease by time on the activity in the urine. There were no significant differences in the dpm g^{-1} measured in the urine during the period from 14 to 96 hr, while the concentrations of [^{14}C] measured at 192, 384 and 1032 hr were significantly lower.

Possible pathways for the excretion of xenobiotics from fish are through the urine, feces, skin and via the gills. The gall bladder has been found to be an important route for the elimination of lipophilic xenobiotics and their metabolites (Lee et al. 1972; Guiney et al. 1977; Roubal et al. 1977; Lech and Bend 1980; Solbakken and Palmork 1981). Solbakken and Palmork (1981) found [^{14}C] in the urine and bile of flounder fed phenanthrene, with the highest amount being found in the bile. The same results were obtained from saithe (Solbakken et al. 1980) and suggested that

the biliary route is the most important route for the excretion of phenanthrene and its metabolites in fish. The low activity of [^{14}C] found in the bile in this study indicates that bile is less important in the elimination of the more water-soluble PHMB and its metabolites. This is in agreement with the findings of Bratt and Hathway (1976) who found no [^{14}C] in the bile of rats fed radioactive PHMB. The PHMB metabolites were excreted via the urine and a small amount through exhaled air. The excretion of [^{14}C] via urine has been reported from the flatfish, *Cithatichthys stigmaeus,* given [^{14}C] naphthalene (Lee et al. 1972). In our study, a higher amount of [^{14}C] was found in the urine than in the bile (Table 2). The elimination of [^{14}C] through the urine is dependent upon the rate of excretion. It is, therefore, difficult to interpret the importance of the excretion of [^{14}C] via urine to the total excretion of radioactivity. Bone and Marshall (1982) give values of urine production in marine teleosts between 0.01 to 0.14 mL hr^{-1} 100^{-1} g body weight. This gives a urine production between 0.7 and 10 mL day^{-1} in a fish of the size used in this experiment. This indicates that a substantial part of the [^{14}C] might be excreted via the urine.

The diffusion of water soluble xenobiotics via the gills may be another important route of excretion (Lech and Bend 1980). The high solubility indicates that the gills and skin may play an important role in the excretion of PHMB with lower molecular weights and metabolites of PHMB in saithe.

A large part of the xenobiotic is probably not absorbed but eliminated via the feces due to the large molecular weight of the compound. This has been shown in rats who excreted 93% of a single dose in the feces. Rats given 20 mg kg^{-1} PHMB absorbed 5.6 % of the given dosage (Bratt and Hathway 1976).

The present investigation shows that PHMB and its metabolites are absorbed and accumulated in low concentrations. The slow rate of elimination of [^{14}C] from muscle indicates a stronger connection with biological molecules here than in the liver. Larger concentrations of [^{14}C] were found in the liver than the muscle, and the rate of elimination from the liver was higher than from the muscle indicating metabolism and excretion from this organ. The concentrations of [^{14}C] in the urine were higher than the concentrations in the bile, indicating that urine is more important in the elimination of this water soluble component.

REFERENCES

Anon (1994) Risk assessment of existing substances. Technical guidance document. European commission. Directorate-General Environment, Nuclear Safety and Civil Protection. XI/919/94.

Anon. (1994) Statistica. Vol 1: General conventions & statistics 1. Statsoft, Ins. Tulsa, OK, USA

Bagenal TB, Tesch FW (1978) Age and growth. In: Bagenal T (ed) Methods for assessment of fish production in fresh water. IBP Handbook No 3, 3rd ed. Blackwell Scientific Publications, Oxford, pp 101-136

Boardman G (1969) A polymeric biguanide for industrial disinfection. Food Techn NZ 4:421-425

Bone Q, Marshall NB (1976) Biology of fishes. Blackie, Glasgow (1982).

Bratt H, Hathway DE (1976) Characterization of the urinary polymer-related material from rats given poly(biguanide-1,5-diylhexamethylene hydrochloride). Makromol Chem 177:2591-2605

Curd FHS, Rose FL (1946) Synthetic antimalarials. Part X. Some aryldiguanide-("biguanide") derivatives. J Chem Soc 11:729-737

Davies A, Field BS (1969) Action of biguanides, phenols and detergents on *Escherichia coli* and its spheroplasts. J Appl Bacteriol 32:233-243

Dexter D, Ficke MB, Decicco BT (1989) Effectiveness of polyhexamethylene biguanide against bacteria of product, clinical and environmental origins. Abstracts of the 89th Annual Meeting of the American Society for Microbiology, A, 122

Guiney PD, Peterson RE, Melancon Jr MJ, Lech JJ (1977) The distribution and elimination of 2,5,2',5'-[^{14}C] tetrachlorobiphenyl in rainbow trout (*Salmo gairdneri).* Toxicol Appl Pharmacol 39:329-338

Larkin DFP, Kilvington S, Kart JKG (1992) Treatment of *Acanthamoeba* keratitis with polyhexamethylene biguanide. Ophthalmology, 99:185-191

Lech JJ, Bend JR (1980) Relationship between biotransformation and the toxicity and fate of xenobiotic chemicals in fish. Environ Health Persp 34: 115-131

Lee RF, Sauerheber R, Dobbs CH (1972) Uptake, metabolism and discharge of polycyclic aromatic hydrocarbons by marine fish. Mar Biol 17:201-208

Neely WB, Branson DR, Blau GE (1974) Partition coefficient to measure bioconcentration potential of organic chemicals in fish. Environ Sci Technol 13:1113-1115

Roubal WT, Collier TK, Malins DC (1977) Accumulation and metabolism of carbon-14 labelled benzene, naphthalene, and anthracene by young Coho salmon (*Oncorhynchus kisutch*). Arch Environ Contam Toxicol 5:513-529

Solbakken JE, lngebrigtsen K, Palmork KH (1984) Comparative study on the fate of the polychlorinated biphenyl 2, 4, 5, 2', 4', 5'-hexachlorobiphenyl and the polycyclic aromatic hydrocarbon phenanthrene in flounder (*Platichthys flesus*), determined by liquid scintillation counting and autoradiography. Mar Biol 83:239-246

Solbakken JE, Palmork KH (1981) Metabolism of phenanthrene in various marine animals. Comp Biochem Physiol 70C:21-26

Solbakken JE, Palmork KH, Neppeiberg T, Scheline RR (1979) Distribution of radioactivity in coalfish (*Pollachius virens*) following intragastic administration of [9-1461 phenanthrene. Bull Environm Contam Toxicol 23: 100-103

Solbakken JE, Palmork KH, Neppelberg T, Scheline RR (1980) Urinary and biliary metabolites of phenanthrene in the coalfish (*Pollachius virens*). Acta Pharmacol et Toxicol 46: 127-132

Veith GD, DeFoe DL, Bergstedt BV (1979) Measuring and estimating the bioconcentration factor of chemicals in fish. J Fish Res Board Can 36: 1040-1048