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Toxicological Effects of Geosmin and 2-Methylisoborneol on Rainbow Trout Hepatocytes

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Geosmin (GM) and 2-methylisoborneol (MIB) are produced by actinomycetes and cyanophytes in the aquatic environment (Gerber 1979; Slater and Block 1983; Persson 1988). These compounds occur in lakes, reservoirs and rivers where GM is more likely to be produced in the water column while MIB is more likely to be produced in sediments (Slater and Blok 1983; Izaguirre et al. 1983). They possess musty, earthy odors at very low thresholds (4 ng/L for GM and 15 ng/L for MIB). Moreover, these compounds tend to accumulate in fish flesh where they can significantly alter its taste and odor. They can also markedly reduce the narcoleptic quality of drinking water (Quatermaine and Anderson 1995). It was recently proposed that zebra mussels indirectly tend to augment the production of these substances because of the accumulation of cyanophytes and actinomycetes in their tissues (Lange and Wittmeyer 1997).

Studies of the toxicological properties of GM and MIB are scarce. Neither substance induced a mutagenic response in the *Salmonella typhimurium* (TA98 and TA100 tester strains) assay up to concentrations reaching cytotoxic levels that are approximately six orders of magnitude greater than the odor threshold concentrations (Dionigi et al. 1993). In another study with sea urchin embryos, the estimated IC_{50} (50 % inhibitory concentration) for GM and MIB were 17 and 69 mg/L respectively (Nakajima et al. 1996), in the same range as the upper concentrations tested by Dionigi et al. (1993). The present investigation was intended to explore the possible cytotoxic effects that these compounds might have on fish hepatocytes. Primary cultures of trout hepatocytes are now well recognized as relevant model systems in toxicological research (Baksi and Frazier 1990). Effects examined were cell viability, vitellogenin (Vg) synthesis (to detect estrogen-like properties), DNA strand breaks, cytochrome P4501A1 (biotransformation of polyaromatic hydrocarbons), and oxidative stress (production of H_2O_2). Based on observed responses, a preliminary index of environmental hazard is proposed for these substances.

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MATERIALS AND METHODS

Hepatocytes were collected from 10-15 cm long rainbow trout (*Oncorhynchus mykiss*) by the double perfusion method (Klauning et al. 1985). Cells were distributed in a 48-well microplate at a density of 1 x 10^6/mL in sterile L-15 medium (at 320 mOsmol/kg H_2O) supplemented with 2% fetal bovine serum, 1000 units of penicillin, 10 mg/L of streptomycin and 25 µg/L of amphotericin B. The cells were exposed to 0.0016, 0.008, 0.04, 0.2 and 1% v/v of geosmin (100 mg/L from Wako chemicals, USA) and 2-methylisoborneol (10 g/L from Wako chemicals, USA) or to concentrations of β-estradiol for the positive control for Vg production (ranging from 0.1 to 10 µM). The wells were sealed with an auto-adhesive film and the plates were incubated for 48 h at 15°C in a humidified incubator. The livers from three female fish were pooled for each experiment.

Cell viability was determined by the propidium iodide (PI) exclusion test (Zucker et al. 1988). Briefly, 50 x 10^3 cells were mixed with 25 µg/mL of PI in phosphate buffered saline (PBS) for 30 min. The cell suspension was then analyzed using a microplate fluorescence plate reader, with dead cells becoming fluorescent at 600 nm during excitation at 485 nm. The fluorescence value was normalized with cell density (absorbance at 600 nm).

The intracellular levels of H_2O_2 in hepatocytes were determined using dichlorofluorescins (Lebel et al. 1992). Briefly, the cells were exposed to 1 µM dichlorofluorescin in PBS for 30 min and the oxidation of dichlorofluorescin into fluorescein was monitored by microfluorimetry at 485 nm excitation and 520 nm emission. The fluorescence value was normalized with cell density (absorbance at 600 nm).

The amount of vitellogenin (Vg) secreted into the extracellular medium was evaluated by the alkali-labile phosphate (ALP) method (Gagné and Blaise 1998; Pelissero et al. 1993). After the 48 h incubation period, 750 µL of the incubation medium is mixed with 500 µL of t-butyl methyl ether for 30 min. The ether phase was then removed and added to 100 µL of 2 M NaOH for 60 min with frequent mixing. The levels of phosphate released into the aqueous phase were then determined by a colorometric assay (Stanton 1968).

Cytochrome P4501A1 activity was measured by following deethylation of 7-ethoxyresorufin (Hahn et al. 1995) with some modifications. Briefly, 1 x 10^5 cells were added to 10 µM of 7-ethoxyresorufin in PBS containing 0.015% triton X-100 for 30 min at 22°C. Afterwards, fluorescence was measured at 590 nm during excitation at 540 nm. Fluorescence intensity was corrected for blank values (t = 0 min) and cell density as measured at 600 nm. Standards of 7-hydroxyresorutin were used to calculate the amount of product formed over time.

DNA strand breaks were determined according to the alkaline precipitation assay (Olive 1988) with some modifications. Briefly, 1 x 10^5 cells were mixed with 2 % sodium dodecyl sulphate containing 10 mM EDTA, 10 mM Tris and 50 mM NaOH for 1 min. Afterwards, one volume of KC1 0.12 M was added and incubation proceeded at 60°C for 10 min. The protein bound genomic DNA was precipitated at 4°C for 30 min followed by centrifugation at 8 000 x g for 5 min. DNA strand breaks remaining in the supernatant were assayed using hoescht dye (Bester et al. 1994) with salmon sperm DNA as the standard.

Cells obtained from the same preparation were exposed to the test samples in four replicates (n=4). The cell effects data were subjected to an analysis of variance where critical differences between exposed and unexposed groups were determined with Dunnett's *t* test. The lowest observable effect concentration (LOEC) in % v/v and the no observable effect concentration (NOEC) in % v/v were then determined. A toxicity threshold (TT) was calculated as follows: TT = $[NOEC \times LOEC]^{1/2}$. Significance was set at $p<0.05$.

RESULTS AND DISCUSSION

When rainbow trout hepatocytes were exposed to concentrations of GM and MIB for 48 h at 15°C a reduction in cell viability was only observed with MIB (Table 1). However, both chemicals incur DNA damage at 0.45 mg/L and 10 mg/L of GM and of MIB, respectively. It thus appears that these odorous compounds can produce DNA strand breaks in fish cells which could lead to harmful mutations (e.g., cancers). In contrast with our results, these compounds had not proven to be mutagenic with the *Salmonella typhimurium* histidine-independence assay (Dionigi et al. 1993). Exposure to GM and MIB also increased ALP levels in the culture medium suggesting that they possess estrogenic-like properties. Normally, Vg production takes place in the liver of female fish and is under the estrogen receptor control, but male fish nevertheless have a functional Vg gene which can be responsive to estrogens (Pelissero et al. 1993, Gillesby and Zacharewski 1998 ; Monosson et al. 1994). Hence, GM and MIB-mediated production of Vg, an energy-rich protein, could reduce energy levels in fish tissues. Exposure to MIB increased EROD activity in hepatocytes. This enzyme system is usually induced by coplanar aromatic hydrocarbons (Monosson et al. 1994) and MIB (an aliphatic alcohol) should not induce cytochrome P4501A1. A possible explanation may be that MIB induces another cytochrome P450 isoform that may display some degree of reactivity towards 7-ethoxyresorufin. It appears that MIB can modulate the phase 1 biotransformation pathway of coplanar aromatic hydrocarbons and, as such, could potentially influence the detoxication (or activation) process of this class of substances under conditions of co-exposure. No significant effects were observed on the relative levels of H_2O_2 in hepatocytes, suggesting that these compounds do not influence the oxidative metabolism of hepatocytes. Toxic

thresholds found with the hepatocyte test were one order of magnitude lower than those found affecting the development of sea urchin embryos (Nakajima et al. 1996) and cytotoxicity to *Salmonella typhimurium* (Dionigi et al. 1993). However, in all cases, the effective concentration to produce an effect far exceeded those found in aquatic environments with severe muddy off-flavor problems.

Table 1. Effects of Geosmin (GM) and 2-methylisoborneol (MIB) exposure on rainbow trout hepatocytes.

Effects [1]	GM TT (mg/L)[2]	MIB TT (mg/L)
Viability	nd[3]	45
DNA damage	0.45	10
EROD activity	nd	45
Vg (Alkali-labile phosphate)	0.45	54
Hydroxyradical levels	nd	nd

1. Rainbow trout hepatocytes were exposed to concentrations of GM or MIB for 48 h at 15°C.
2. TT is the toxicity threshold at which a positive effect is observed. TT = (NOEC x LOEC)$^{1/2}$ where LOEC is the lowest observable effect concentration and NOEC is the no observable effect concentration.
3. nd : no effect detected at the highest concentration tested, i.e., 1 mg/L for GM and 100 mg/L for MIB.

In view of further results shown in Table 2, we can offer some idea of the relative hazard posed by GM and MIB. Based on octanol/water partition coefficients, we can derive bioconcentration factors for GM and MIB in fish (Neely et al. 1974). In addition, if we extrapolate the concentration of GM and MIB in fish tissues, the relative hazard index range for GM and MIB is 0.006-0.03 and 8×10^{-7}- 3×10^{-4}, respectively. Since these values are < 1, the likelihood of harm toward fish by these substances would be very low. Although preliminary hazard assessment of these substances suggests that GM and MIB are relatively harmless following acute exposure, the long term health risk on fish populations remains to be evaluated. For example, MIB was found to alter electrocardiograms of trouts at lower concentrations found in the river (Kawamura et al. 1995). Moreover, diverse types of interactions (e.g., chronic exposures with other contaminants throughout the life cycle of organisms and biomagnification) are likely to occur in an aquatic ecosystem. For example, consumers of zebra mussels could be more exposed to these substances since these bivalves ingest microorganisms which produce GM and MIB (Lange and Wittmeyer 1997). Finally, it is not at all

improbable that the sublethal effects observed could eventually lead to long term (chronic) consequences at the individual level. Clearly, further investigations will be required to address these concerns.

Table 2. Preliminary hazard assessment.

	Geosmin	2-Methylisoborneol
Octanol/water partition coefficient[1] ($\log K_{ow}$)	3.7	3.1
Bioconcentration factor	2.13	1.80
Reported levels in the St. Lawrence River and the Great Lakes[2]	20-100 ng/L	25-150 ng/L
Hazard index[3]	0.006-0.03	8×10^{-7} - 3×10^{-4}

1 .From Pirbazari et al. 1992.
2. From Ridal et al. 1999; Vogel et al. 1997; Palmentier et al. 1998; Brownlee et al. 1984.
3. The index is defined as the derived-concentration in tissues / mean effective concentration of the compound. A ratio > 1 is considered as potentially harmful. For GM an effective concentration of 0.45 mg/L was used from the DNA damage and Vg tests (Table 1). For MIB an effective concentration of 32 mg/L was used which is the average of the TT for DNA damage and VC tests from Table 1.

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