Sublethal effects of manganese on the carbohydrate metabolism of *Oreochromis mossambicus* after acute and chronic exposure

I. Barnhoorn,* J.H.J. van Vuren and H.H. du Preez

Department of Zoology, Rand Afrikaans University, P.O. Box 524, Aucklandpark, 2006 South Africa

Carbohydrate metabolism variables of *Oreochromis mossambicus* were investigated after acute and chronic sublethal manganese exposure. The sublethal concentrations were determined from the LC_{50} value of manganese. After the exposures, the fish were carefully netted and blood was drawn from the caudal aorta. The differences in the values of carbohydrate metabolism variables of exposed fish were measured against control values and statistically analysed to prove statistically significant differences in variable values, caused by the metal pollutant ($P < 0.05$). The results obtained showed changes in the carbohydrate metabolism variables (glucose, lactate, glucose-6-phosphate dehydrogenase and pyruvate kinase concentrations). These alterations are produced as a result of increased levels of cortisol and catecholamines, as well as hypoxic conditions. The latter induce hyperglycemia and increased lactate levels. Hypoxia may be a result of the damaging effect of manganese on the gills after exposure. The enzymes involved in the carbohydrate metabolism are sensitive to metal exposure and therefore enzyme concentrations fluctuated after the exposure to manganese. Enzyme function plays an important role in the catalysing of chemical reactions in an organism and the disturbance thereof could lead to death. Fish enzyme levels are therefore important biomarkers in the event of metal pollution in a water source.

* Author to whom correspondence should be addressed.

Introduction

Information concerning the sublethal effects of metals form an integrated part of the ecosystem health assessment programmes and of procedures followed to develop water quality guidelines for the environment (Roux, Badenhorst, Du Preez & Steyn 1994). Generally, concentrations that produce sublethal chronic effects are lower than those that produce more readily observable acute effects, such as death (Rand & Petrocelli 1985). Toxic effects of pollutants (e.g. metals), and therefore the health and well being of organisms (such as fish), after exposure were mainly evaluated by the above-mentioned acute test when the death of the organism was the only criteria (Larsson, Haux & Sjöbeck 1985). The possible sublethal effects of pollutants were generally neglected. Biochemical/physiological processes, however, would be affected or even stop to function normally before the onset of death. The sublethal effects of pollutants are usually biochemical/physiological in nature, since most of these pollutants affect the basic level of organisation that is the subcellular level in an organism (Thomas 1990). Data on the sublethal effects of pollutants would aid in the identification of pollution before drastic changes occur in the natural population. Information of sublethal effects on fish populations is therefore needed to develop realistic water quality guidelines and ecosystem health assessment.

Manganese has been identified as a pollutant, according to its general toxicity from literature data, as well as concentrations detected in the abiotic and biotic components of the Olifants River, Mpumalanga (Seymore 1994). With this available information, it was decided to expose fish to sublethal manganese concentrations to establish physiological effects of this potentially harmful metal on carbohydrate metabolism. Carbohydrate metabolism provides (1) energy, (2) precursors for synthetic reactions and (3) reduction/oxida-

tion mechanisms for converting the precursors to appropriate intermediates or final products. The major product of carbohydrate digestion is glucose (Walton & Cowey 1982). An increase in glucose and lactate concentrations in the blood of fish are typical indicators of stress (Hattingh 1972). Most of the carbohydrates are stored in the liver as glycogen. Additionally, smaller amounts are also evident in the muscle glycogen (Heath 1987; Voet & Voet 1990). Carbohydrates are transported as blood glucose in the fish's body, which is regulated by the hormones glucagon and insulin (Hepher 1988). The dynamics of carbohydrate metabolism are greatly influenced by stressors. A stressor is any environmental change that influences the homeostatic balance in an animal (Heath 1987). Firstly, the chromaffin cells are affected via the hypothalamus. These cells are in the walls of the cardinal veins and in some cases the anterior kidney. When affected, these cells release adrenaline and noradrenalin, which promote both the conversion of liver glycogen into blood glucose, as well as the use of glucose by the muscle. An increase in blood lactic acid concentrations also occurs frequently with stress (Heath 1987). In cases where long periods of stress situations are present, the release of glucocorticoids (cortisol) increase as a response to the adrenal corticotrophic hormone (ACTH) from the adenohypophysis (Donaldson 1981; Heath 1987; Pottinger & Moran 1993). The sensitive character, as discussed above, of blood glucose and blood lactate to detect the presence of pollutants is the reason why these parameters were chosen as variables in this toxicological study, exposing the fish to sublethal concentrations of manganese.

The determination of enzyme activities in serum has become an important diagnostic tool in toxicological studies. Various factors may be responsible for an alteration of serum enzyme levels that is (1) the altered enzymatic activities in the organs of the fish; (2) changed iso-enzymes of different

catalytic properties under the conditions of the test; (3) the leakage of enzymes into the serum; and (4) altered elimination rate of enzymes out of the serum (Sauer & Haider 1977; Giesy, Versteeg & Graney 1988). The enzymes involved in carbohydrate metabolism are sensitive to chemical stress, which may result in a decrease in key enzymes involved in glycolysis (e.g. pyruvate kinase and glucose-6-phosphatase) and the Krebs cycle (Heath 1987). Since all chemical reactions in cells are essentially catalysed by enzymes, the action of a foreign chemical in the cell almost always involves disturbances in enzyme function (Heath 1987; Weis & Weis 1991).

The data obtained from this study were implemented to develop and expand the existing water quality index (WATER2) into the current RAUWATER. This index could be an important tool in the monitoring of metal ions in the aquatic environment.

Materials and methods
Experimental procedures

Mozambique tilapia, *Oreochromis mossambicus*, was chosen as test organism because (1) they occupy a position within a food chain leading to man; (2) they are widely available and abundant; (3) they are amenable to laboratory testing; (4) they are genetically stable, thus uniform populations can be tested; (5) they are available throughout the year, to name but a few criteria (Rand & Petrocelli 1985; Gill & Pant 1987; Heath 1987). The fish were obtained from the University of Zululand, Empangeni, KwaZulu-Natal. They were transported in an aerated 1000 litre aluminium transport tank filled with water from the dam where the fish were sampled. Sodium chloride (0.3 to 1%) and dinitrofurazane (20 g per 100 *l* water) were added to the water in order to reduce the stress levels and bacterial infection that could possibly be sustained during transport (Carmichael, Tomasso, Simco & Davis 1984). At the aquarium, fish were maintained and acclimated in recirculating systems with borehole water for approximately three months, prior to experimentation. The physico-chemical characteristics of the borehole water are compiled in Table 1. After 72 hours, the fish were fed daily on commercial trout pellets (50% protein). The fish were acclimated at 23±1°C. Photoperiod and temperature can influence the behaviour and metabolism of fish. Water temperature was kept constant at 23±1°C and the photoperiod was regulated with a timer to produce 12:12 hour day : night cycles (Van Vuren 1986; Grobler, Van Vuren & Du Preez 1989). After the acclimation period of three months, the fish were transferred to the

flow-through exposure system (Figure 1) which was based on a concept from Sprague (1969). *O. mossambicus* were exposed to sublethal concentrations of manganese chloride under controlled laboratory conditions for 96 hours (short-term exposure) and 28 days (long-term exposure) respectively. Sublethal concentrations (Slc) were calculated as percentages of the manganese LC_{50} concentration as determined by Seymore (1994). For the sublethal exposures 10%, 15% and 20% of the LC_{50} were used (Table 2).

During the exposure experiments, control groups were also set-up. The control fish were kept under the same laboratory conditions as the exposure groups, without the addition of manganese to the borehole water. These enzymatic parameters from the control fish set a baseline of values to detect the effects of manganese on fish (Sprague 1973). Ten *O. mossambicus* were exposed to sublethal manganese concentrations for 96 hours. The actual manganese concentrations [Mn$^+$] in the water were measured by means of atomic absorption spectrophotometry. Water samples were taken from the exposure system and aqua-regia digestion was carried out (Van Loon 1980) (Table 2).

Blood sampling

The exposed fish were carefully netted to minimize stress (Blaxhall & Daisley 1973). Immediately after the fish were

Table 1 Summary of values of selected water quality variables of borehole water

pH	7.65
Temperature (°C)	23
Ammonium (NH$_4$, mg/l)	0.04
NO$_3$ + NO$_2$ as N (mg/l)	1.97
Fluoride (F, mg/l)	0.6
Total alkalinity as CaCO$_3$ (mg/l)	48
Sodium (Na, mg/l)	3.5
Magnesiom (Mg, mg/l)	7.5
Silicon (Si, mg/l)	10
Phosphate (PO$_4$, mg/l)	0.025
Sulphate (SO$_4$, mg/l)	16
Chloride (Cl, mg/l)	5.5
Potassium (K, mg/l)	2.05
Calcium (Ca, mg/l)	12
Conductivity (I c, mS/m)	15.1
Total dissolved solids (TDS, mg/l)	114.5

Table 2 Normal and observed sublethal manganese concentrations administered to the water during exposures

Exposure groups	Control groups	A	B	C	Control groups	D	E	F
Exposure times	96 hours	96 hours	96 hours	96 hours	28 days	28 days	28 days	28 days
[Mn] g/l	0	0.172	0.259	0.345	0	0.172	0.259	0.345
MnCl$_2$ Applied g/l	0	0.594	0.99	0.119	0	0.594	0.99	0.119
[Mn$^+$] In water AAS g/l	n\d	0.109	0.172	0.196	0	0.137	0.184	0.232

A = Sublethal concentration 10 (acute exposure); B = Sublethal concentration 15 (acute exposure); C = Sublethal concentration 20 (acute exposure); D = Sublethal concentration 10 (chronic exposure); E = Sublethal concentration 15 (chronic exposure); F = Sublethal concentration 20 (chronic exposure); AAS = atomic absorption spectrophotometry
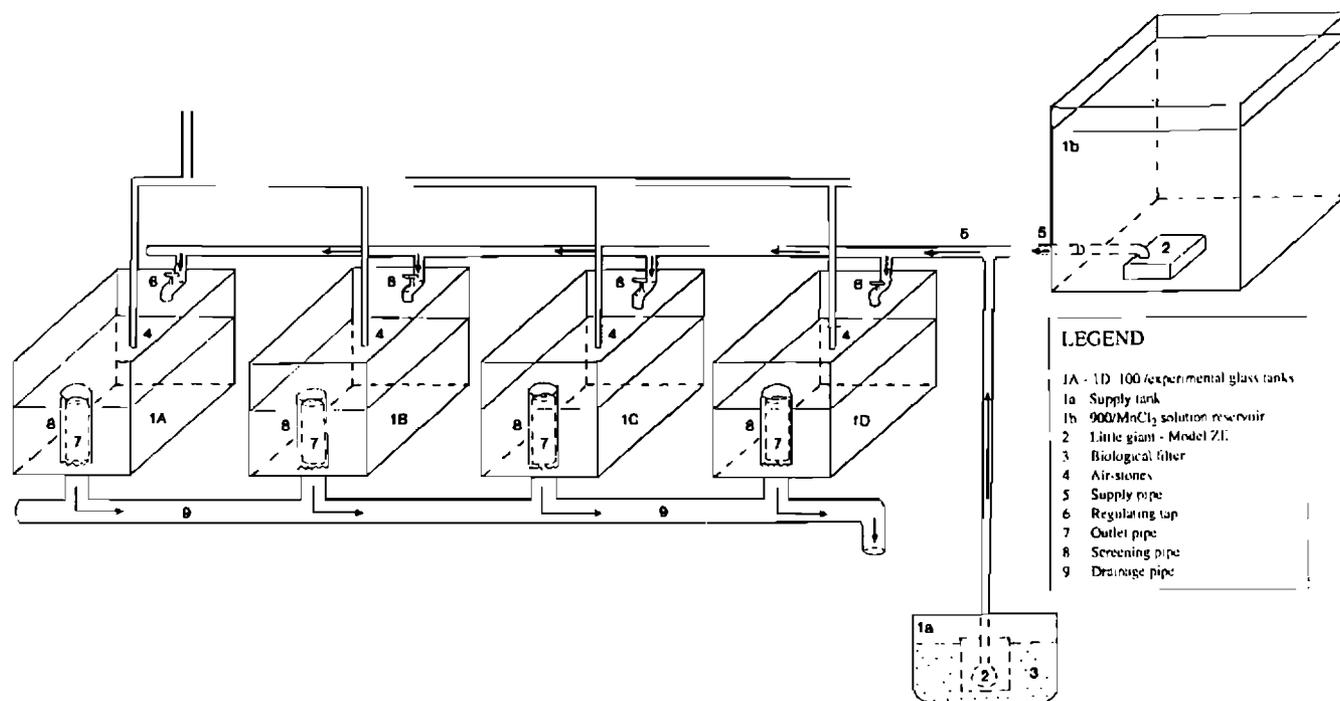
Figure 1 Schematic diagram of the experimental flow-through system (Tanks 2A–2D and 3A–3D were set up in the same way, but are not illustrated)

caught, blood was drawn from the caudal aorta, using a 1 ml plastic heparinised (5000 U/ml) syringe supplied with a 26G needle (Larsson, Bengtsson & Svanberg 1976). The area where the blood was drawn, was wiped dry to avoid contamination with mucus and water (Blaxhall & Daisley 1973). In order to prevent haemolysis, minimal suction power was exerted to the syringe (Klontz & Smith 1968).

Measurement of metabolic and the carbohydrate metabolism-enzyme activities

Both the glucose and lactate concentrations were determined colormetrically by means of Boehringer-Mannheim-test combinations and a Hitachi 150-20 Spectrophotometer. Pyruvate kinase (PK) was determined according to the method of Gutmann and Bernt (1974). The PK activity was determined as the production of nicotinamide adenine dinucleotide (NAD) in the lactate dehydrogenase-coupled reaction at 340 nm. The glucose-6-phosphate dehydrogenase (G6P-DH) activity was determined by means of a Boehringer-Mannheim-test combination.

Data processing

Data were processed on an IBM compatible computer utilising a Statgraphics statistical programme. Independent Student's T-tests were performed to prove probability hypothesis. Differences in mean values were accepted as being statistically significant if $p < 0.05$.

Results

The plasma glucose concentration increased significantly ($p < 0.005$) after the three short-term exposures (Figure 2A-C) and after the 0.259 g/l long-term exposure (Figure 3D-F). There was a significant increase ($p < 0.005$) in the plasma lactate

concentration after the 0.345 g/l short-term exposure (Figure 2C). Although there were no significant changes in the glucose-6-phosphate dehydrogenase concentration after the short-term exposures (Figure 2A-C), there was a significant increase ($p < 0.05$) after the 0.259 g/l long-term exposure (Figure 3E). The plasma pyruvate kinase concentration resulted in a significant decrease ($p < 0.005$) after the 0.345 g/l short-term exposure (Figure 2C).

Discussion

The literature that reviews the toxicity of manganese, is limited. However, the few documented studies done showed that sublethal concentrations of manganese have an impact on the carbohydrate metabolism and haematology of fish (Nath & Kumar 1987; Wepener, Van Vuren & Du Preez 1992). These studies include pH 1 as a variation factor. Although the pH-factor was not included during this study, manganese affected the levels of the monitored variables. In previous studies it was found that the glucose concentrations increased during starvation of fish as well as stressful conditions (Giesy et al 1988). The use of blood glucose measurements appears to be a sensitive, reliable indicator of environmental stress in fish (Silbergeld 1974. Cassilas & Smith 1977). The increase in the blood glucose concentrations (hyperglycemia) after the short-term exposures and 0.259 g/l long-term exposure could be as a result of increased levels of cortisol and catecholamines (Nath & Kumar 1987; Wepener 1990). Catecholamines may deplete glycogen reserves in stressed fish by stimulating glucogenolysis and gluconeogenesis (Heath 1987). This hypophysis-adrenal response was also observed in Salmo gairdneri exposed to stressful conditions (Cassilas & Smith 1977) and in nickel exposed Colisa fasciatus (Chaudhry & Nath 1985). The significant increase in the lactate concentration after the
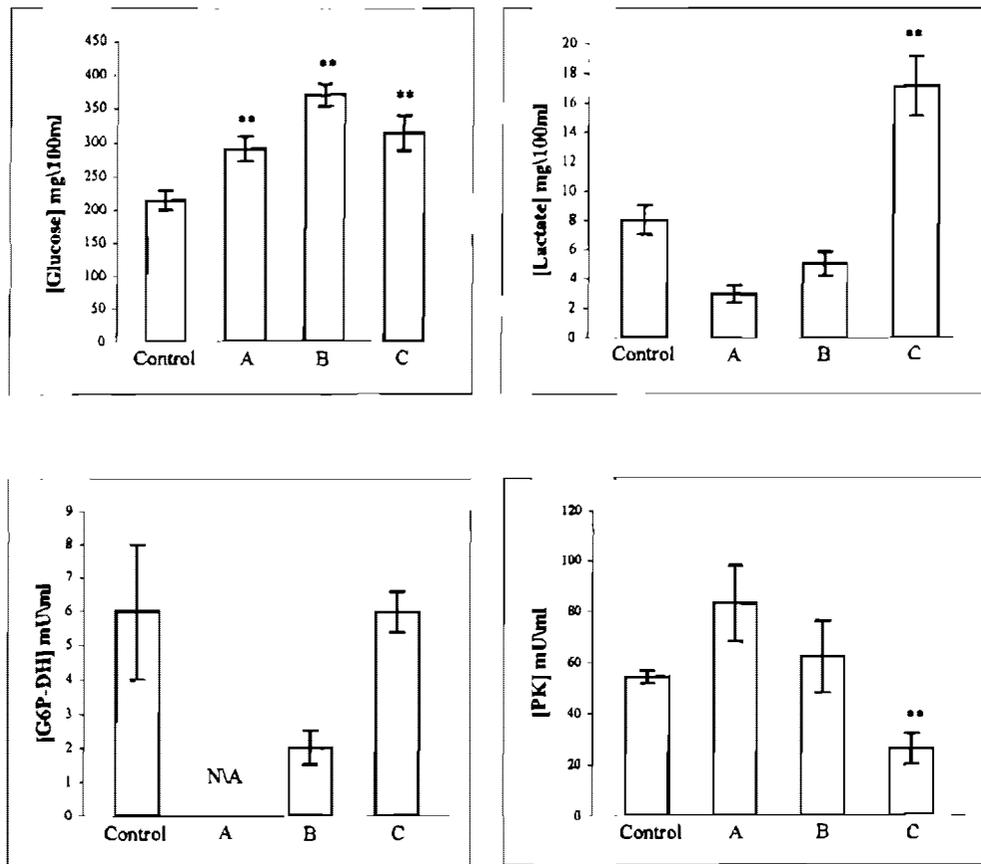
Figure 2 The mean glucose, lactate and enzymatic values of *O. mossambicus* after the short-term manganese exposures at $23\pm1°C$ ($\bar{X} \pm S.E$; $n = 10$). ** = $p \le 0.005$. A = 0.173 g\l exposure; B = 0.259 g\l exposure; C = 0.345 g\l exposure; N\A = not available

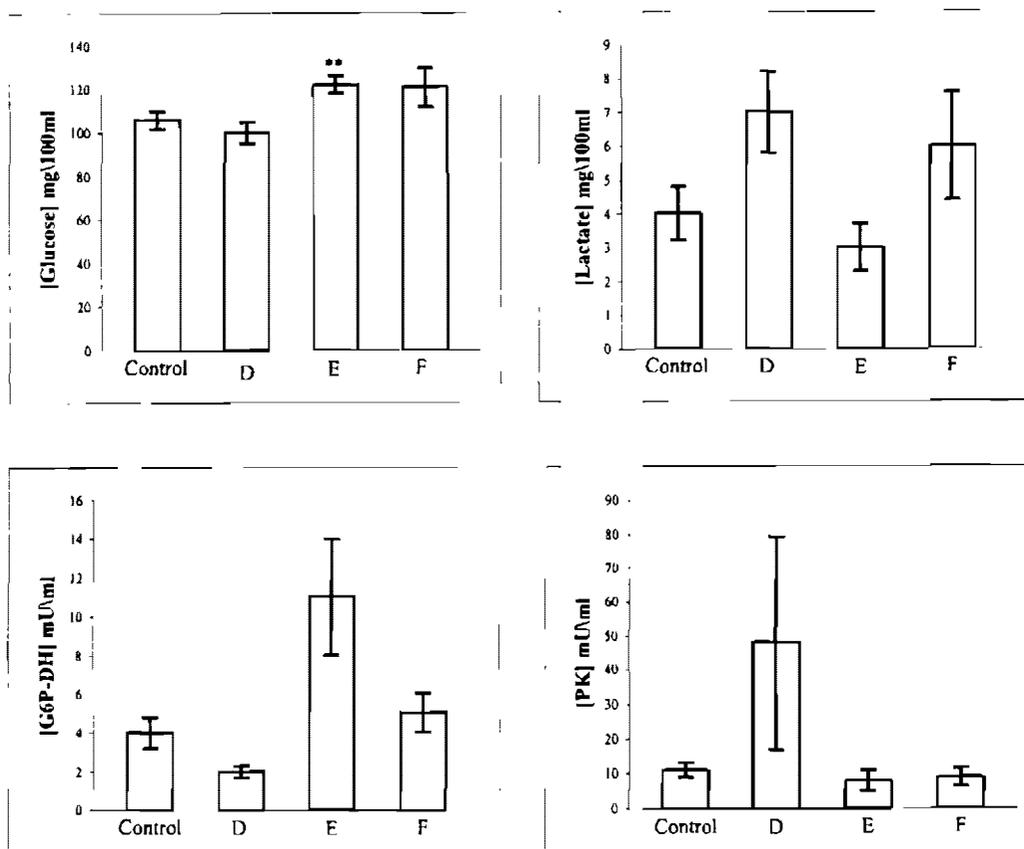**Figure 3** The mean glucose, lactate and enzymatic values of *O. mossambicus* after the long-term manganese exposures at $23\pm1°C$ (X $\pm$ S.E.; $n = 10$). ** = $p \le 0.005$. D = 0.173 g\l exposure; E = 0.259 g\l exposure; F = 0.345 g\l exposure

0.345 g/l short-term exposure may be as a result of hypoxia of the tissue and the blood (Heath 1987). This increase in anaerobic metabolism may occur due to the damaging effects of the manganese on the gills of exposed fish (Nath & Kumar 1987; Wepener 1990). During anaerobic metabolism lactate is produced through homolactic fermentation. Much of this lactate is exported from the muscle cells and transported by the blood to the liver. In the liver it can be reconverted to glucose (Arms & Camp 1987). Lactate is transported via the bloodstream to the heart and liver under normal conditions. These aerobic tissues catabolise lactate (through respiration) or convert it back to glucose (Voet & Voet 1990).

The enzymes in the extracellular cavities have no metabolic function, but they are sensitive indicators of the cellular changes, which are caused by pathological conditions. The presence of cellular enzymes in the extracellular cavities is due to a loss of energy in the cells (Friedel, Diederichs & Lindena 1979). Inhibition of the cation-pump by toxicants causes swelling of the cells and increased levels of intracellular calcium. This results in increased membrane permeability due to increased membrane vesicles. Enzymes then diffuse out of the cells into the blood (Heath 1987). Alternatively the enzymes diffuse through the capillary membrane or via the lymph to the blood (Friedel et al. 1979). An increase of the cellular enzyme in the plasma can be interpreted as a decrease in the activity of that enzyme in the tissue it is active (Gerlach 1968). During glycolysis, PK couples the free energy of phospho-enol-pyruvate hydrolysis to the synthesis of ATP to form pyruvate. Pyruvate can be converted to glucose through gluconeogenesis (Voet & Voet, 1990). The decrease in the plasma PK levels after the 0.345 g/l short-term exposure may be an indication of PK activity elsewhere. This means the possibility of an increase in glycolysis in the muscle of fish (Knox, Walton & Conwey 1980).

Additional energy rich compounds are produced in the pentose phosphate pathway. G6P-DH oxidises glucose-6-phosphate to fructose-6-phosphate which is converted to pyruvate during glycolysis, which is reconverted to glucose. The main toxicological site of action by manganese are the gills (Wepener 1990). In the gills additional energy is needed to regulate the osmotic balance in fish. Therefore there may be an increase in G6P-DH activity in the gills to produce the additional energy for osmoregulation (Bhaskar & Govindappa 1985). This is probably the reason for the increase in G6P-DH activity after the 0.259 g/l long-term exposure.

According to this study it is evident that manganese is harmful to fish even in small concentrations. Although Hellawell (1986) suggested that manganese does not have much significance as a pollutant, there were a variety of significant changes in the levels of the variables measured. Manganese is one of the first metals to increase in concentration in acidified waters (Bendell-Young & Harvey 1986). The influence of industrial wastes and mining effluents on the rivers increase the manganese concentrations in water (Moore & Ramamoorthy 1984). Exceeded manganese concentrations may lead to altered physiological functions. As manganese acts as a stressor in the aquatic environment and may affect the survival of the organisms involved, it is thus important to monitor manganese levels to avoid hazardous events.

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