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Whole Effluent Toxicity Testing with *Oncorhynchus mykiss* (Walbaum 1792): Survival and Behavioral Responses to a Dilution Series of a Mining Effluent in South Africa

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Abstract. Survival, behavioral early warning responses to, and behavioral effects of a complex effluent from Richards Bay Minerals in Natal, South Africa, were studied using rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) as a biosensor. Three replicates of eight juvenile fish were exposed to a dilution series of the effluent (0, 5, 10, 20, 50, 75, 100%) and the behavioral pattern (ventilation, locomotion) was measured online with quadropole impedance conversion. Survival decreased with increasing effluent concentration. Before death, *O. mykiss* coloration darkened, demonstrating skewed bodies subsequently after death. Decreased activity and increased ventilation combined with increased ventilation frequency were found within the first 2 h of exposure at ≥96-h LC_{50} value of 10% effluent concentration indicating early warning responses. During 4 days of exposure, activity decreased and ventilation increased further at concentrations around the 96-h LC_{50} value indicating toxic effects.

At present, control and surveillance of industrial wastewater discharges depend mainly on physicochemical measurements, which, due to the discontinuity of sampling, do not always detect detrimental effluent discharges in time and cannot predict toxicity of complex wastes. According to the triad approach for the evaluation of stream ecosystem quality, chemical analyses (environmental chemistry) should be combined with biological analyses of the community structure (aquatic ecology) and additionally with toxicity assessments, preferably *in situ* (toxicological effect biomonitoring) (Monda *et al.* 1995). A biological monitoring system uses living organisms as sensors. Changes in biomarkers of the test species are related to toxicant stress. Biomarkers on different biological organization levels can act as effective early warning sentinels to ensure protection of the integrity of ecosystems (Depledge and Fossi 1994).

Online biological monitoring can not only integrate synergistic or antagonistic effects of chemicals in the effluent, but can also provide an advance warning of insiduous toxic conditions (Morgan and Kühn 1989). With the development of biological early warning systems (BEWS) based on *in situ* online biomonitoring, whole effluent toxicity testing (WET) below discharge sources is possible using selected indicator species and selected nondestructive biomarkers, *e.g.* behavioral or physiological parameters for the detection of toxic responses or effects. Behavioral parameters have the advantage of being highly integrative (Scherer 1992) *i.e.* they are based on neurological-biochemical reactions on the suborganism level, but they also show ecological consequences on the supraorganism level, such as altered abundance due to altered drift as consequence of altered locomotion (Janssen *et al.* 1994; Lagadic *et al.* 1994).

Several fish monitors based on different techniques such as ultrasonic, video, conductivity, bioelectrical signals, and rheotaxis have been developed (Juhnke and Besch 1971; Morgan 1975; Miller 1977; Van der Schalie *et al.* 1979; Van Hoof 1980; Gruber and Cairns 1981a, 1981b; Thompson *et al.* 1982; Cairns and Garton 1982; Van der Putte *et al.* 1982; Evans and Johnson 1984; Smith and Bailey 1988; Lorenz *et al.* 1995; Thomas *et al.* (1996). They have the advantages of using a broad spectrum sensor, which is at least as sensitive as human beings to a wide range of chemicals (Evans and Wallwork 1988).

The aim of this study was to evaluate the toxicity of a complex mining effluent using the common fish species *Oncorhynchus mykiss* as sensor in a novel online biomonitoring system. Direct behavioral responses as well as toxic effects after short-term exposure were measured.

Materials and Methods

Study Site

The test water was taken from the effluent of Richards Bay Minerals, Natal, South Africa. Richards Bay is situated in a wetland area close to the Umhlatusi Lagoon and the Umlazi Nature Reserve at the coast of the Indian Ocean about 60 km south of Lake St. Lucia and about 140 km north of Durban. Toxic effluents of the industry complex might detrimentally affect the wetlands and the coastal ecosystems. The water quality data of the effluent reveal great variations in metal concentrations and salts combined with slightly acidic water (Table 1).

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Table 1. Chemical characterization of the effluent water from Richards Bay Minerals

Parameters	June 96	July 96	Oct. 96	Nov. 96
pH	6.6	6.5	5.9	6.0
Anion (meq/L)	6.7	—	7.8	6.9
Cation (meq/L)	6.5	—	7.5	6.3
Ion-balance (%)	−1.4	—	−2.0	−5.1
PO_4 (mg/L)	0.01	0.15*	0.01	0.01
SO_4 (mg/L)	153	92*	193	137
NO_3-N (mg/L)	0.4	—	0.6	0.4
Cl (mg/L)	103	—	118	120
HCO_3 (mg/L)	35	—	20	40
Na (mg/L)	53	39	69	72
K (mg/L)	6.6	11	7.6	4.5
Mg (mg/L)	12.8	9.6	14.1	17.8
Ca (mg/L)	60	110	61	31
Al (mg/l)	—	0.07	—	—
Cd (mg/L)	—	<0.002	—	—
Cu (mg/L)	<0.014	<0.014	0.009	<0.014
Fe (mg/L)	0.001	0.02	0.261	0.001
Mn (mg/L)	0.0002	0.17	0.092	0.12
Zn (mg/L)	0.0247	0.166	0.371	0.128
Pb (mg/L)	—	0.08	—	—

The metals Ti, Co, Cr, Mo, Ni, and V were below the detection limits of the ICP-AES instrument; * : elements instead of salts measured

Test Species

Hatchery-reared larvae of rainbow trout *O. mykiss* Walbaum 1792 of a medium size of 25 mm (±5 mm) were obtained from the fish hatchery at the Department of Ichthyology and Fischeries Science at Rhodes University. Rainbow trout were held in flow-through systems with tapwater (10°C) being fed twice a day with powderized commercial fish food. Rainbow trout was used as test species due to its worldwide occurrence, its ecological key role as top predator in stream ecosystems, and its use in standard toxicity bioassays in the EEC, USA, and Canada.

Experimental Design

The fish were kept in groups of eight organisms in aerated tanks filled with 10 L tapwater at 15 ± 1°C in darkness for 24 h before treatment. The fish were fed twice a day with food described above, and the water was renewed daily. Test solutions were prepared from mining effluent water diluted with tapwater to the following concentration levels: control (tapwater), 5, 10, 20, 50, 75, and 100% effluent. The fish were placed individually in the test chambers and acclimated to the test water for 10 min to allow for settlement after the handling stress. Afterwards the behavioral pattern of eight fish was recorded online for 24 s in intervals of 10 min for the following 2 h. For each concentration level, three experiments with eight fish each were performed. After these initial measurements of possible direct responses to the toxic effluent, the fish were kept for 4 days under the described conditions in their respective test solutions. Survival was recorded daily. After 4 days, the behavioral pattern of the survivors of each concentration level was recorded as described above to reveal possible toxic effects of the effluent.

Behavioral Biomonitor System

The principle of the biomonitor was based on quadropole impedance conversion technique (Gerhardt *et al.* 1994). The fish moved freely in a cylindrical flow-through test chamber made of plexiglass pipe (3.2 cm in diameter, 9 cm long, 144.7 ml volume) between two interweaved coils of platinum wire electrodes at the opposite chamber walls. The current-carrying pair of electrodes produced an alternating current of 50 kHz and 1 mA over the chamber. The non–current-carrying pair of electrodes measured changes in the impedance, which were generated by movements of the organisms in the electrical field. This biomonitoring system differed from the WRcIII Fish Monitor (Evans and Wallwork 1988) and the bioelectronic monitor (Microvolt Oy, Helsinki) (Laitinen *et al.* 1996) by separating the measurements of changes in the electrical field from the generation of the field in two different sets of electrodes.

The electrical signals generated by the impedance converter were processed in a Mac LCIII computer using software written in LabView 2.1.1. The analysis algorithm was based on the power spectrum for the impedance variations. The spectrogram was then analyzed with a pattern-matching procedure described elsewhere (Gerhardt *et al.* 1998). In the present study, time spent on locomotory activity and ventilation was analyzed by observing at preselected broad ranges of amplitudes and frequencies, generated by the organisms' movements in the test chamber.

Different types of signals described different types of behaviors. (1) Ventilation/opercular movement/mouth-opening was a monofrequent behavior characterized by the frequency range between 2.8 and 4.7 Hz and one small amplitude (0.02–0.2 V depending on position in the chamber). (2) Locomotion/swimming was characterized by a range of frequencies, mostly below 2.8 Hz and several amplitudes, mostly above 0.7 V. The following pattern could be observed: a strong body movement followed by smaller movements with the fins.

The preselected frequency ranges were too large to detect other typical fish behaviors, such as coughing (one of the most sensitive parameters for rapid environmental stress in fish) (Biro and Hughes 1985; van der Schalie *et al.* 1988), ventilation depth, and heart rates (Laitinen *et al.* 1996).

Statistics

Survival data were analyzed by linear regression after probit transformation and 96-h LC_{50} values with 95% confidence intervals were calculated (Weber 1986). Behavioral data followed skewed normal distributions and were analysed by two-factor (concentration levels, three replicate experiments of each eight organisms) ANCOVAs with time as covariate (12 subsequent recordings in 2 h) for all parameters, *i.e.* selected frequency and amplitude ranges typical for different behaviors. In case of significant results *post-hoc* Tukey-Kramer tests revealed significant pairwise comparisons. Comparisons of the behavioral patterns of the first 2 h with those after 4 days of exposure were done by two-factor (concentration levels, time of measurement) ANCOVAs followed by Tukey-Kramer tests.

Results

Survival

The handling stress of transferring the fish and measuring behavior in the test chambers did not affect the survival of the fish, as they were able to survive in the test chambers for at least 10 h. Handling stress of ≤30 min seemed to have no severe effects on the stress parameters ventilation frequency and lysozyme activity in *O. mykiss* (Moeck and Peters 1990). No changes in overall activity, however, increased high frequency and low amplitude behavior such as ventilation was observed towards the end of the test period of 10 h (Figure 1).

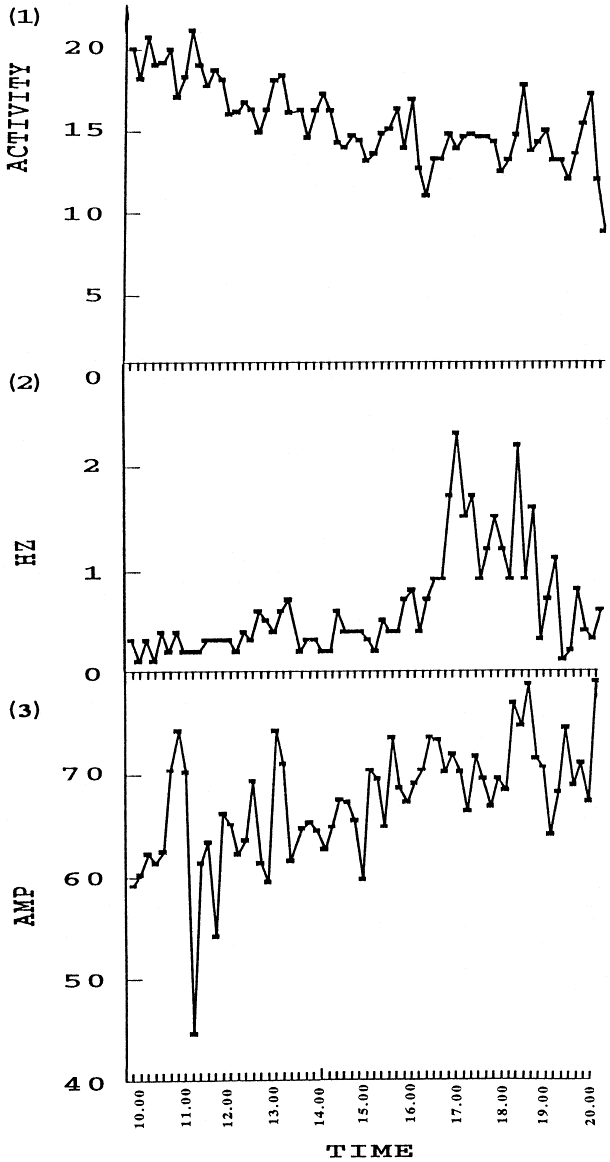


Fig. 1. Behavior of juvenile rainbow trout (*Oncorhynchus mykiss*) in the test chambers during 10 h continuous recording with impedance conversion. (1) Time spent on activity(s), (2) Time spent on high frequency behavior ($3.8 < x < 4.7$ Hz), and (3) Time spent on low amplitude behavior (<0.2 V)

Over an exposure period of 4 days in the tanks, survival of the fish decreased with increasing effluent concentration (Figure 2). The 96-h LC_{50} value was 10% effluent concentration (95% CI: 0–20% effluent) and the regression equation $y = 0.048\times + 4.5$ was significant ($p = 0.001$; $F = 60.25$, $df = 1$) with an R^2 of 0.9. A few hours before death, exposed fish showed darkened coloration and inward-crooked bodies after death.

Direct Behavioral Responses

The overall activity of the fish did not decrease linearly with increasing effluent concentration. However, significant differences were found ($p < 0.001$; $F = 27.4$; $df = 6; 2$). The lowest activity levels were found at 20% and 50% effluent, slightly above the range of the 96-h LC_{50} (Figure 3).

The most dominant frequency range in trout behavior was $1.9 < x < 2.8$ Hz, indicative for locomotion in case of high amplitudes, and for ventilation in case of low amplitudes (Figure 4). At 20 and 50% effluent concentration, the frequency range of $1.9 < x < 2.8$ Hz decreased in favor of the next higher frequencies in the range of $2.8 < x < 3.8$ Hz, typical for ventilation ($p < 0.001$; $F = 22.1$; $df = 6; 2$). This indicated increased time spent on ventilation at increased ventilation frequency at concentrations above the 96-h LC_{50} value. The frequency range <1.9 Hz, typical for locomotion, increased slightly at concentrations $\geq$75% effluent ($p < 0.001$, $F = 10.8$; $df = 6; 2$). The frequency range $3.8 < x < 4.7$ Hz, the least frequently shown behavior, decreased slightly at $\geq$50% effluent concentration ($p < 0.001$; $F = 7.6$; $df = 6; 2$). The most dominant amplitudes were <0.2 V, typical for the small movements of ventilation. At concentration levels $\geq$20% effluent, a significant increase in these movements could be observed (Figure 5) ($p < 0.001$; $F = 5.7$; $df = 6; 2$). High amplitude movements such as turning in the chambers occurred very rarely and were more expressed at lower concentration levels $\leq$20% effluent ($p < 0.001$; $F = 20.4$; $df = 6; 2$).

Toxic Behavioral Effects After 96 Hours

The behavior of the survivors at concentration levels $\leq$50% effluent showed decreased overall activity for the control and $\leq$10% effluent after 4 days of exposure ($p < 0.001$; $F = 14.1$; $df = 2; 1$) (Figure 6). The lowest activity at 10% effluent coincided with the 96-h LC_{50} value. No significant differences between direct (0–2 h) and delayed (96 h) effects in the concentration levels 0, 10%, and 50% effluent were found for the frequency ranges $\leq$1.9 Hz and $\leq$2.8 Hz due to high interindividual variation in the behavior. The frequency range $2.8 < x < 3.8$ Hz, typical for ventilation, revealed increased ventilation at 10%, however decreased ventilation at 50% effluent concentration after 4 days compared to respective direct responses ($p < 0.001$; $F = 7.4$; $df = 2; 1$). Amplitude ranges $0.2 < x < 0.7$ V ($p < 0.008$; $F = 4.9$; $df = 2; 1$), $0.7 < x < 2$ V ($p < 0.0001$; $F = 16.2$; $df = 2; 1$) and $2 < x < 8$ V ($p < 0.001$; $F = 29.3$; $df = 2; 1$) showed decreased values after 4 days in all concentration levels.

Discussion

Chemistry of the Effluent

The mining effluent of Richards Bay Minerals contained a series of metals in varying concentrations and slightly acidic water. The pH values were around 6, thus not toxic for *O. mykiss.* Effects of low pH on different trout species were only seen at pH $<$ 5 as impaired locomotion and feeding (Delonay *et al.* 1996). Brook trout showed avoidance at pH $\leq$ 5.5 (Pedder and Maly 1986) or at pH $<$ 5 and 0.2 mg A1/L (Gagen *et al.* 1994). Impairment of swimming performance due to increased oxygen requirements was found for rainbow trout at pH 5.2

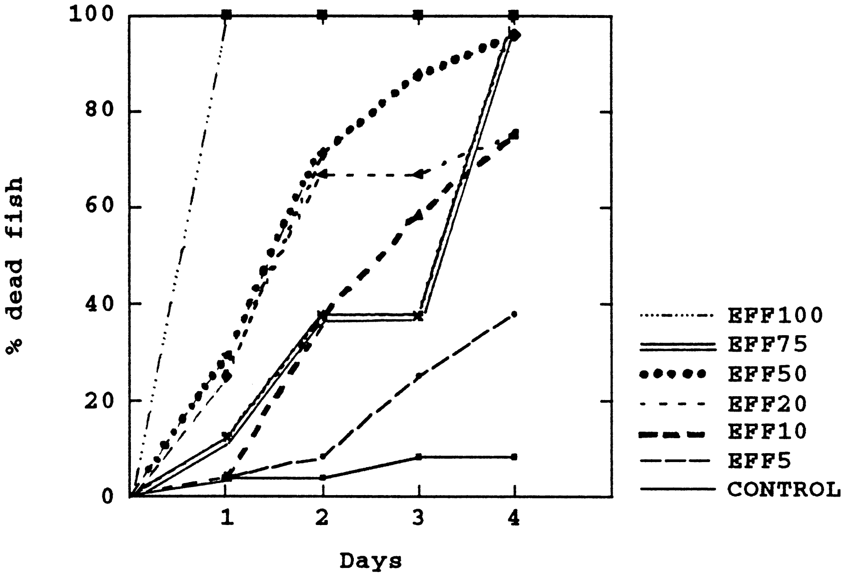


Fig. 2. Mortality of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to different concentrations (control, 5, 10, 20, 50, 75, 100%) of Richards Bay Minerals effluent for 96 h in 10-L tanks

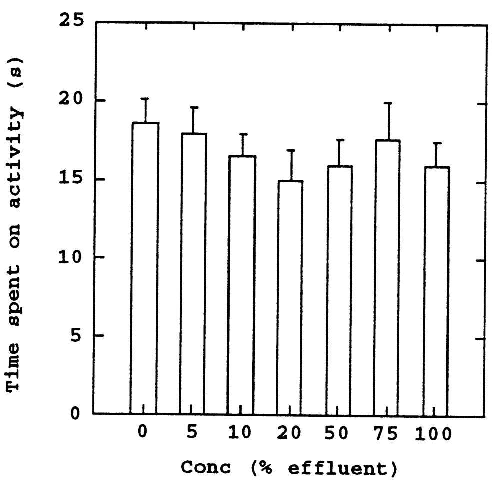


Fig. 3. Direct responses of juvenile rainbow trout (*Oncorhynchus mykiss*) to different concentrations (control, 5, 10, 20, 50, 75, 100%) of Richards Bay Minerals effluent: time spent on activity(s)

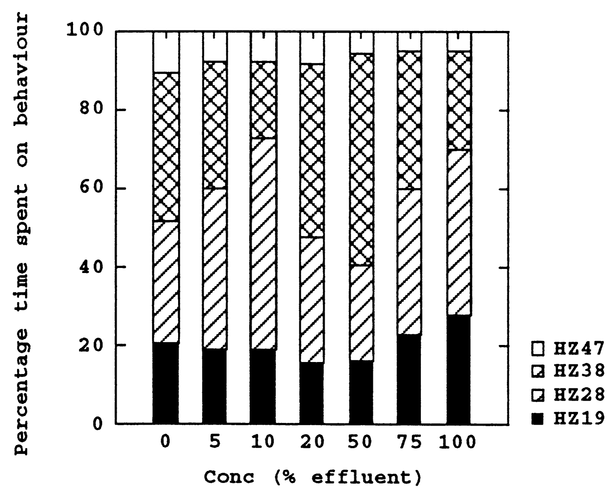


Fig. 4. Direct responses of juvenile rainbow trout (*Oncorhynchus mykiss*) to different concentrations (control, 5, 10, 20, 50, 75, 100%) of Richards Bay Minerals effluent: percentage time spent on different behaviors as characterized by selected frequency ranges such as (1) HZ19: $<$1.9 Hz, (2) HZ28: $1.9 < x < 2.8$ Hz, (3) HZ38: $2.8 < x$ 3.8 Hz, and (4) HZ47: $3.8 < x < 4.7$ Hz

(Wilson *et al.* 1994). The copper values of the effluent water were above the background levels for freshwater (Jørgensen *et al.* 1991), but in the range of those causing effects in rainbow trout such as impaired feeding and locomotion at 0.01 mg/L or decreased survival at 0.04 mg/L (96-h LC_{50}) (Blaxter and Ten-Hallers Tjabbes 1992). Sublethal effects of copper at 5 µg/L have been reported for salmonids (Flemming and Trevors 1989); Atlantic salmon avoided concentrations of 2.3 µg Cu/L and 53 µg Zn/L (Henry and Atchison 1991). However, increasing salinity prevented detrimental effects of Cu on *O. mykiss* due to competition of ions at the gill membrane (Verbost *et al.* 1987). The metals Zn and the Pb could reach 10 times higher concentrations in the effluent than the background levels reported for freshwater (Jørgensen *et al.* 1991). Al concentrations in the effluent were around the threshold values reported for toxic effects on aquatic life (Weatherly *et al.* 1990). The concentration levels of the salts in the effluent fell within the range of concentrations measured in west African lakes, rivers, and reservoirs; however *O. mykiss* occurs only as introduced species in highland streams of low salinity (Frempong 1995).

Survival

The 96-h LC_{50} value of 10% of the mining effluent for *O. mykiss* is very low compared to 96-h LC_{50} values for single metals such as Pb (1.17 mg/L at pH 6.7–7.3) or Cu (0.06 mg/L) for the same species (Atchison *et al.* 1987). Sodium chloride concentrations of 12 mg/L affected the "critical thermal maximum" of red shiners (*Notropis lutrensis*) (Beitinger and McCauley 1990). Thus, mixtures of several metals and salts

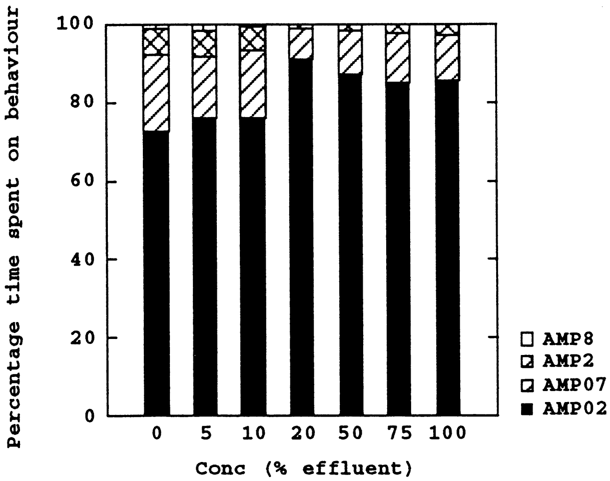


Fig. 5. Direct responses of juvenile rainbow trout (*Oncorhynchus mykiss*) to different concentrations (control, 5, 10, 20, 50, 75, 100%) of Richards Bay Minerals effluent: percentage time spent on different behaviors as characterized by selected amplitude ranges such as (1) AMP02: < 0.2 V, (2) AMP07: $0.2 < x < 0.7$ V, (3) AMP2: $0.7 < x < 2$ V, and (4) AMP8: $2 < x < 8$ V

combined with slightly acidic pH could have had synergistic detrimental effects on the survival of *O. mykiss* in the present experiment.

Direct Behavioral Responses

Within 2 h of exposure, juvenile rainbow trout reacted to the mining effluent with decreased overall activity as well as increased time spent on ventilation and increased ventilation frequency. These findings correspond with those of other authors and indicate that *O. mykiss* is a sensitive and fast-reacting biosensor for chemical stress. Behavioral responses of fish toward various toxicants were found within 30 min to ≥35 µgKCN/L (Thomas *et al.* 1996), within 40 min at concentration levels of 10–25% of the LC_{50} (Baldwin *et al.* 1994), within 1 h at concentrations of ≤50% of 96-h LC_{50} (Evans and Wallwork 1988), and within 1 h toward several organic compounds (Kaiser *et al.* 1995). Within 4 h of exposure to ≥0.1 mg/L carbofuran, goldfish responded with increased burst swimming behavior (Saligo *et al.* 1996). Avoidance responses of fish were measured at 1:3,000 of LC_{50} of a metal plating effluent containing Ni and Cr (Hadjinicolaou and Spraggs 1988) and at 0.1–5% of the LC_{50} for various toxicants using *O. mykiss* as test species (Little *et al.* 1993). Rainbow trout and brook trout avoided metal mixtures at concentration levels known from impacted rivers (Delonay *et al.* 1996). Calcium salts have been reported to induce consistent avoidance in sticklebacks, however sodium salts had no effects on avoidance of sunfish (*Lepomis cyanellus*) (Beitinger 1990). Due to elevated salt concentrations in the mining effluent, *O. mykiss* might have shown osmotic stress expressed as increased ventilation as early warning response. Changes in ventilation and locomotion have also been reported to be fast and sensitive indicators for pollution stress (Gerhardt 1995, 1996) in crustaceans and insects. This indicates a universal behavioral response that can be used in biological early warning systems.

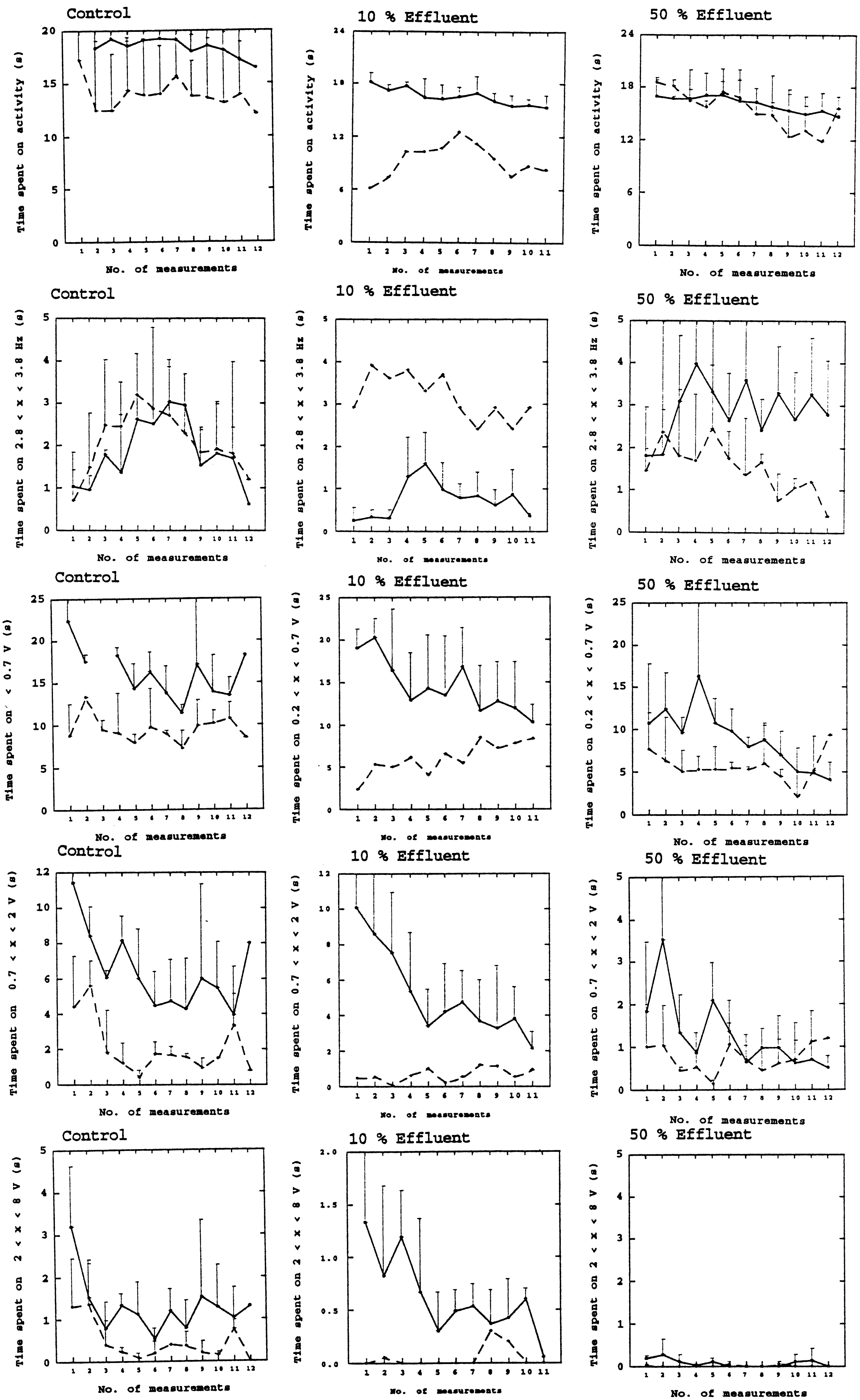
Behavioral Toxic Effects After 96 Hours

Due to acclimation to the small 10-L tanks, activity decreased slightly in all exposure conditions over the 4-day period. This corresponds with observations by van der Schalie *et al.* (1988). Decreased overall activity of *O. mykiss* measured as direct response within 2 h persisted after 4 days of exposure at concentration levels around the 96-h LC_{50} value. This corresponds with other findings, where spontaneous swimming activity of rainbow trout was inhibited by a variety of organic toxicants after 1 day (Kennedy *et al.* 1996) or after 4 days of exposure (Little *et al.* 1990).

Ventilation ($2.8 < x < 3.8$ Hz) increased at 10% effluent concentration after 4 days of exposure, but decreased at 50% effluent concentration. Such "opposite reactions" were also mentioned for rainbow trout exposed to organic chemicals: increased swimming at low concentrations and decreased swimming performance at high concentrations (Beitinger and McCauley 1990). Thomas *et al.* (1996) found decreased electric organ discharges (EOD) in a tropical electrical fish at low KCN concentrations followed by increased EOD at higher KCN levels. With increasing toxicant concentrations, variability of individual responses often increases combined with a dichotomous response pattern, such as either increased ventilation or increased locomotion in the mayfly *Adenophlebia auriculata* exposed to copper (Gerhardt and Palmer 1998) or rainbow trout exposed to 1,3,5-TNB (van der Schalie *et al.* 1988). Opposite behavioral reactions at different toxicant concentrations might be due to different underlaying mechanisms, *e.g.* increased locomotion at low concentrations might be an avoidance reaction, whereas decreased swimming activity at higher concentrations might be an acclimation to the toxicant stress by changes in resource allocation. Moreover, reactions to toxicant mixtures might provoke such responses, as different components in the toxic cocktail have different response thresholds for different types of behavior of the test organism.

Mechanisms of Toxicity and Ecological Consequences

Metal ions seem to interfere with olfactory receptor binding sites of fish (Blaxter and Ten Hallers-Tjabbes 1992). Such reactions occur within a few hours and provoke avoidance responses in fish, which can be expressed as (1) increased time spent on ventilation as a trial to remove the ions from the binding sites due to increased water flow at the interface membrane/water, as observed in this study within the first hours of exposure; (2) increased swimming activity as a trial to escape from the contaminated place (*e.g.* brook trout: ≥6 µg Cu/L) (Blaxter and Ten Hallers-Tjabbes 1992); or (3) decreased locomotion as a trial to be spooled away from the toxic site with the drift.


Control
10 % Effluent
50 % Effluent
Time spent on activity (s)
No. of measurements
Control
10 % Effluent
50 % Effluent
Time spent on 2.8 < x < 3.8 Hz (s)
No. of measurements
Control
10 % Effluent
50 % Effluent
Time spent on 0.2 < x < 0.7 V (s)
No. of measurements
Control
10 % Effluent
50 % Effluent
Time spent on 0.7 < x < 2 V (s)
No. of measurements
Control
10 % Effluent
50 % Effluent
Time spent on 2 < x < 8 V (s)
No. of measurements

Changes in gill ventilation of fish often indicate osmoregulatory and ionregulatory defects caused by pollutants or salts. Gradual transfer of *O. mykiss* to seawater revealed changes in liver carbohydrate metabolism (Soengas *et al.* 1995), increased alkaline phosphatase activity in the intestine, and decreased enzyme activity in the kidney (Gasser and Kirschner 1987). Changes in ionregulation at the gills of *O. mykiss* transferred to seawater have been measured as increased Na^+- and K^+-ATPase activity (Pagliarani *et al.* 1991) and decreased HCO_3^-- and Ca^{2+}-ATPase activity (Fuentes *et al.* 1995). Increased salinity led to increased energetic costs and metabolic rates as well as decreased growth of *O. mykiss* (Morgan and Iwama 1991).

Decreased swimming performance as observed in the present study might be due to altered resource allocation, *i.e.* increased energy demands for other physiological processes result in less energy reserves for locomotion. Such other physiological processes might be osmo- and ionregulation, induction of stress proteins, or increased energy turnover (Triebskorn *et al.* 1997). Brown trout exposed to polluted surface water containing pesticides and heavy metals showed significant decreased swimming velocity, morphological and physiological changes in the liver and induction of stress proteins (Triebskorn *et al.* 1997). Changes in swimming performance can have ecological consequences, such as impaired migration or altered predation, and thus effects on growth and reproduction of the fish (Little *et al.* 1993). Different types of behavioral responses to toxicants have been distinguished: type 1 caused by narcotic chemicals, type 2 caused by neurotoxins (*e.g.* lead, cadmium), and type 3 caused by metabolic disruptors (Little *et al.* 1993). As in the present experiment, juvenile rainbow trout reacted with decreased activity, increased ventilation, darkened coloration, and deformed bodies after death, a type-1 or type-2 response might have been provoked by the effluent of Richards Bay Minerals.

In summary, the slightly acidic effluent water of Richards Bay Minerals with its varying metal and salt concentrations was toxic for juvenile *O. mykiss.* Directly after exposure the fish reacted with decreased locomotion and increased ventilation as early warning responses, indicating osmotic and ionregulative stress. After 4 days of exposure, 10% effluent concentration killed 50% of the fish and the behavioral changes in the surviving fish persisted.

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Fig. 6. Comparisons of selected behaviors of juvenile rainbow trout (*Oncorhynchus mykiss*) shown within the first 2 h of exposure (full line) with respective behavior shown after 96 h (dashed line): (1) time spent on activity(s), (2) time spent on $2.8 < x < 3.8$ Hz, (3) time spent on $0.2 < x$ 0.7 V, (4) time spent on $0.7 < x < 2$ V, and (5) time spent on $2 < x < 8$ V

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