Screening the Toxicity of Ni, Cd, Cu, Ivermectin, and Imidacloprid in a Short-Term Automated Behavioral Toxicity Test with *Tubifex tubifex* (Müller 1774) (Oligochaeta)

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Running Head: Screening Toxicity Test with *Tubifex tubifex*

ABSTRACT

A new automated online toxicity test for screening of short-term effects of chemicals is presented using the freshwater oligochaete *Tubifex tubifex* in the Multispecies Freshwater Biomonitor™ (MFB). Survival and locomotory behavior of the worms were observed during 24 h of exposure to metals (Cd, Cu, Ni), pesticides (Imidacloprid), and pharmaceuticals (Ivermectin). The LC_{50} values revealed increasing toxicity in the following order: Ni (> 100 mg/l) < Cu (15.2 mg/l) < Cd (4.9 mg/l) < Ivermectin (1.8 mg/l) < Imidacloprid (0.3 mg/l). The EC_{50} for locomotion showed a similar order of increasing toxicity: Ni (86 mg/l) < Cu (3.8 mg/l) < Ivermectin (2.0 mg/l) < Cd (1.1 mg/l) < Imidacloprid (0.09 mg/l). Toxicity was dependent on both concentration and exposure time. This could be demonstrated in 3d response models and proven in the statistical analysis showing a significant interaction term (C x T) for the experiments with Cu and Ni. *T. tubifex* proved to be very tolerant, but even then behavioral responses were more sensitive than mortality for Cu, Cd, and Imidacloprid.

Key Words: multispecies freshwater biomonitor™, locomotion, EC_{50}, LC_{50}.

INTRODUCTION

Freshwater Oligochaeta have long been used in aquatic biomonitoring, particularly in the classification of the trophic state of lakes and large rivers. They are used as indicators of sediment pollution based on saproby and oxygen depletion, *e.g.,* the ratio of *Limnodrilus hoffmeisteri* to other tubificid worms (Aston 1973; Chapman and Brinkhurst 1984), or the ratio of tolerant oligochaetes to chironomids (Wiederholm 1978; Gerhardt 2002).

Tubifex tubifex is a tube-forming endobenthic freshwater oligochaete, capable of tolerating low oxygen levels. Adult *T. tubifex* are known to survive, grow, and reproduce under anoxic conditions (Famme and Knudson 1985). *T. tubifex* feeds on organic sediment particles, such as detritus, fungi, bacteria, and algae. Head-first in a sediment tube, the worms ventilate with their tail in order to create a flow of water, bringing oxygen and food particles into the tube (Guerin and Giani 1996).

T. tubifex have been used in toxicity assessment with several endpoints, *e.g.,* survival, burrowing behavior and avoidance, reproduction, and population growth (Lotufo and Flegger 1996). Bettinetti *et al*. (2003) performed a whole sediment toxicity test of 10 days with *T. tubifex* considering survival, growth, cocoon deposition and development as test parameters to evaluate organic micropollutants, where growth and reproduction proved the most sensitive endpoints. A similar test design has been used by Reynoldson (1994) for analysing toxic stress of environmental sediment samples from the Great Lakes, where effects on reproduction and mortality were correlated to metal contents of the sediments. Egeler *et al.* (2001) studied the effect of contaminated *T. tubifex* on the bioaccumulation of hexachlorobenzene by fish, proving that contaminated prey significantly enhances bioaccumulation by predators compared to non-contaminated food.

Behavioral responses are positioned at the whole-organism level, reflecting biochemical changes and leading to ecological consequences (Lagadic *et al.* 1994; Janssen *et al.* 1994). In addition to their integrative nature (linking different biological organisation levels) and ecological relevance, behavioral responses are non-destructive and amongst the first and most sensitive responses to occur (Warner 1967). Behavioral parameters are thus optimal for cost-effective, ecologically relevant rapid toxicity assessment, biological early warning systems, and online biomonitors where repeated measurements are needed. Behavioral aquatic ecotoxicology is receiving more attention and application since quantitative and automatic behavioral

measurement systems have been developed (Gerhardt 1999; Dell'Omo 2002; Gerhardt 2007). For example, behavioral avoidance has proven to be a sensitive endpoint in earthworm toxicology under exposure to crude oil and 2,4,6-Trinitrotoluene (Schaefer 2003). Leynen *et al.* (1999) described by video technique the retraction behavior of Tubificidae into the sediment, when exposed to metal salts. Petry (1989) described an automated early warning system based on change of spontaneous motility of a *Tubifex tubifex* aggregation recorded by video; however, this system is meant for water bioassays only. A retraction into the aggregate mass of worms was followed by active escape.

The aims of this study were to develop a simple test procedure for a cost-effective, fully automated rapid behavioral aquatic toxicity screening test with *T. tubifex* and to test whether *T. tubifex* is a sensitive test species to a series of chemical substances.

MATERIALS AND METHODS

Maintenance of *T. tubifex*

Three hundred g of adult *T. tubifex* was commercially acquired and kept in a 25 L aquarium containing a thin layer of ashed sand (5 mm height with sand of 0–2 mm grain size), an aquarium filter for continuous recirculation of dechlorinated tapwater (1.78 mmol/l, 20^{0}C, 12 h:12 h photoperiod) and additional aeration with an airstone. The water was replaced every two days, where also food (1 teaspoon of powderized TetraMin/TetraPhyll 50/50%) was added. *T. tubifex* could be maintained for up to 6 weeks, then the organisms started to lose color and vitality. Aggregation to large "carpets" in the aquarium was achieved within 15 min after each change of water. Only a few solitary worms were found in the thin sediment layer.

Choice of Test Substances

The test substances covered different chemical classes, such as metals, pesticides, and pharmaceuticals, *i.e.,* chemicals with different sites and modes of action. Three metals were chosen, Ni and Cu (essential) and Cd (toxic). Nickel is one of the essential metals, *e.g.,* found in several enzymes and is regarded moderately toxic to fish dependent on water hardness (WHO 1991). For the experiments, $NiCl_2(6H_2O)$ was used to test the following nominal concentrations of Ni: 0, 1, 5, 10, 25, 50, 75, 100 mg/l. Copper is one of the best investigated essential metals concerning uptake and toxicity towards aquatic fauna. Copper is an essential micronutrient,

necessary for a wide range of metabolic processes, but at high doses copper sulfate and other copper compounds are effective algicides, and usually free copper ions are the lethal agent by inhibiting sodium uptake at the sodium channels (Flemming and Trevors 1989, Grossell *et al.* 2002). Copper(I)chloride was used in the following nominal concentrations of Cu: 0, 0.01, 0.05, 0.1, 1, 5, 10 mg/l. Cadmium is highly toxic to wildlife; it has severe sublethal and lethal effects at relatively low environmental concentrations. It affects respiratory functions, enzyme levels, muscle contractions, growth reduction, reproduction, and survival (ATSDR 1999). Cadmium(I)chloride-hemipentahydrat was used in the following nominal concentrations of Cd: 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 mg/l.

Imidacloprid is a widely applied neurotoxic chloro-nicotyl insecticide, based on blocking the nicotinergic neuronal pathways. The blockage leads to accumulation of AChE, resulting in the animal's paralysis. Imidacloprid is moderately toxic to aquatic fauna, has a medium water solubility (0.5 g/l, 20 ^{0}C) and its half-life in water is around 1 month (Extoxnet 2006). Imidacloprid was used in the following nominal concentrations: 0, 0.0025, 0.005, 0.0075, 0.01, 0.05, 1, 10 mg/l. Ivermectin is an anti-parasite medication against intestinal worms, mites, and lice, used for animals and humans, especially to treat river blindness in the tropics. Water solubility is 4 mg/l. Ivermectin acts on insects by potentiation of GABA-ergic neural and neuromuscular transmission, resulting in paralysis (Inchem 2006). Ivermectin was used in the following nominal concentrations: 0, 0.1, 0.5, 1 mg/l without solvent (methanol) and 5, 10 mg/l with solvent (methanol) together with an additional solvent control.

Experimental Setup

The natural spontaneous locomotory behavior of unexposed single worms as well as groups of worms was recorded in cylindrical flow-through test chambers (single worms: 4 cm length, 1 cm diameter; groups of 25 worms: 4 cm length, 2 cm diameter) sealed at both ends with nylon nets (250 um) and exposed in aerated dechlorinated tapwater for 1 h. Recording with the Multispecies Freshwater Biomonitor™ and simultaneous visual observation allowed for the following classification of two types of behavior: locomotion of single worms (0.5–3 Hz), and ventilation of a group of worms in an aggregate (1.0–1.5 Hz).

For all experiments, artificial water (0.714 mmol/l, Ca: 11.5 mg/l, Mg: 8.0 mg/l, Na: 11.6 mg/l, K: 6.2 mg/l, Cl: 13.5 mg/l, SiO_2: 31.7 mg/l, SO_4: 8.1 mg/l, HCO_3: 71 mg/l, pH: 7) and the

following metal salts were used: Ni(II)chlorid hexahydrat, Copper(I)chlorid, Cadmium(I)chlorid-hemipentahaydrat, all at 98% purity. Methanol (0.01 %) was used as a solvent for Ivermectin at 5 and 10 mg/l, and additionally as a solvent control.

Experiments were performed in polyethylene aquaria filled with 500 ml artificial water, under static conditions without areation, in three replicates with each containing three chambers (4 cm long, 2 cm diameter), with 25 worms per chamber. The chambers were connected to the Multispecies Freshwater Biomonitor™ (MFB). The MFB recorded every 10 min a trace of 4 min (locomotory behavior) after an initial acclimation time of 30 min. The MFB is based on a non-optical recording principle, so called quadropole impedance technique, recording quantitatively the whole behavioral pattern in a fully-automated way on real-time basis (Gerhardt *et al.* 1994; Gerhardt 1999). The organism moves free in a flow-through test chamber, containing two pairs of stainless steel electrode plates at the inner chamber walls, one pair of electrodes creating a high frequency alternating current, the other pair recording impedance changes in the electrical field, caused by the organism's movements. These signals were then analysed by a discrete Fast Fourier Transform in order to get a histogram of the occurrence of different signal frequencies for the whole recording period (Gerhardt *et al*. 1998).

Survival was checked after 24 h by visually counting the worms. The 24 h experiments were performed at room temperature (20^{o}C) and with a 12 h:12 h photoperiod.

Statistical Analyses

Twenty-four h LC_{50} and EC_{50} values after 6, 12,18, 24 h for locomotory activity (% time spent on locomotion as 50% of the control (control = 100%)) were calculated according to the standard probit method for survival and a log-linear regression for behavior (Weber 1986). Statistical comparisons between treatments were done with arcsine ($x^{½}$) transformed data (% survival, % time spent on behavior) using one factor repeated measures ANOVA for time periods (ti) of ti 1– 4 (each 6 hrs with 36 subsequent records of behavior) followed by Tukey's tests. In order to avoid pseudoreplication the data for three chambers in one aquarium were averaged before statistical analysis.

Regression analysis consisted of two approaches: 1) a 3d model (concentration, exposure time, response) as described in Gerhardt *et al.* (2005) using the following formula: $L = \alpha + \beta C + \gamma T + \delta(C \times T)$ with L: Locomotion, C: Concentration levels, T: Exposure Time. The parameters

α, β, γ, and δ were estimated by an iterative convergence quasi-Newton process with a loss function (observed-predicted)^2. For the sake of easy interpretation higher order polynomials were not applied. 2) Bivariate regressions were fitted for each ti-x period describing the relationship between response and concentration of toxicant. Whereas the regression analysis is a modelling approach allowing for predictions, the statistical analysis revealed the detailed significant differences in the recorded original data, hence providing complementary and more in-depth arguments for the observed overall regression models.

RESULTS

Calibration of *Tubifex tubifex* Behavior

Two types of movement patterns could be discerned, locomotion of single worms and aggregation in group exposures. The worms attach with the head parts to each other and move their tails synchronously with a homogenous regular frequency of 1.0–1.5 Hz, resembling the ventilation behavior of other aquatic invertebrates (Figure 1).

Survival

Significant concentration-dependent decreases in survival could be observed for all test substances except Ni, which was not toxic in the concentration range of 0–100 mg/l (Table 1 part A). Probit regressions revealed mostly significant equations as a basis for the LC_{50} calculation. The LC_{50} values revealed increasing toxicity in the following order: Ni (> 100 mg/l) < Cu (15.2 mg/l) < Cd (4.9 mg/l) < Ivermectin (1.8 mg/l) < Imidacloprid (0.3 mg/l). The statistical comparison of survival in different concentration levels for each substance showed no concentration-dependency for Ni, whereas for all other substances there was a tendency that survival was lower at higher concentrations (*e.g.*, for Cd, ICP) compared to lower concentrations and the controls (Table 1 part B).

Behavior

All substances affected the spontaneous locomotion of *T. tubifex* in a concentration and time dependent manner, when fitting a 3d concentration-time-response model as described in the Methods section (Table 2). The detailed 2d regression between locomotion and concentration levels showed decreasing response slopes with increasing exposure time for most of the

substances, and significant regressions between toxicant concentrations and response. This means that locomotion decreased with increasing concentration of contaminant and exposure time. The detailed statistical analysis supported the regression analysis in the way that significant differences in spontaneous locomotion were found to be dependent on concentration levels (mostly for high concentration levels) for all substances, and also dependent on exposure time (*i.e.,* overall decreasing locomotion with increasing exposure time); however, a significant interaction term (CxT) was only found for the metals (Table 3). In the test with Ivermectin, the solvent methanol had already a significant influence on locomotion (control > solvent, 5 and 10 mg Ivermectin/l; $F(3,8) = 11.8$, $p = 0.003$) additional to the general concentration level effect.

The EC_{50} values for locomotion showed a similar order of increasing toxicity: Ni < Cu < Ivermectin < Cd < Imidacloprid (Table 4, Figure 2). Moreover, for Cu, Cd, and Imidacloprid the behavioral responses after 24 h of exposure were more sensitive than the effect on survival.

DISCUSSION

Tubifex tubifex proved to be a very tolerant aquatic test organism regarding the LC_{50} values for metals after 24 h of exposure, such as 4.9 mg/l for Cd, 15.2 mg/l for Cu and > 100 mg/l for Ni. A comparison of LC_{50} data from the literature shows a wide variation in test results for *T. tubifex* exposed to Cd, which might be due to differences in test design, source, and health state of the animals, food sources, water quality, *etc*. The present study was at the more sensitive side of the published range of LC_{50} 24 h: 1.2–450 mg Cd/l (AQUIRE 2006). For other freshwater oligochaetes the LC_{50} 96 h was 0.15 mg Cd/l for *L. variegatus* (Hickey and Martin 1995), 1.7 mg Cd/l for *Nais* sp. (Rehwoldt *et al.* 1973) and 56 mg Cd/l for *T. tubifex* (Rathore and Khangarot 2002). However, Thorp and Lake (1974) found a lower value for Cd-toxicity in *T. tubifex* of 0.027 mg Cd/l.

Other benthic invertebrates, such as mayflies, seem to be more sensitive, *e.g.,* for *Baetis tricaudatus* the LC_{50}-96 h was 1.6 µg Cd/l (Irving *et al*. 2003), for *Hyalella azteca* a value of 3.8 µg Cd/l was found at low Ca levels and 55 µg Cd/l at 150 mg Ca/l (Jackson *et al.* 2000). Crustaceans seem to be particularly sensitive to Cd (LC_{50} 96 h amphipods: 62 uµg Cd/l), whereas aquatic insects are more tolerant (LC_{50} 96 h: 840–233,000 µg Cd/l (Wong 1987)). During short-term exposure (96 h) to Cd in acutely toxic concentration levels, automaty of the caudal ends was observed in *T. tubifex* (Bouche *et al.* 2000). This is supported by a field observation from

metal (Cu, Pb) contaminated sites, where *T. tubifex* had abnormal caudal ends (missing or regenerating), combined with the finding that posterior parts contained more metals than anterior parts (Bouche *et al.* 1999). Sublethal levels of Lindane™ caused automaty and sediment avoidance in *T. tubifex* during 72 h exposure to a sediment-based toxicity test design (Meller *et al.* 1998). Other sublethal parameters, such as feeding behavior in the chironomid *Glyptotendipes pallens*, resulted in effects at > 0.1 mg Cd/l (Gerhardt 1993). The EC_{50} 28 d for number of eggs of oligochaetes were 2.7 mg Cd/l and 8.4 mg Cu/l (Chapman *et al.* 1999); both values, were also found for behavioral responses in *T. tubifex* in the present study.

Compared to Cd, the LC_{50} values for *T. tubifex* after 24 h of exposure were much higher when exposed to the essential metals Cu and Ni. Generally, the LC_{50} 48 h varied from 5 µg Cu/l (*Daphnia* sp.) to 64 mg Cu/l (Trichoptera) (Gerhardt 1993). LC_{50} 96 h values between 30–6,000 µg Cu/l have been reported for fish, 5–86 µg Cu/l for *Daphnia* sp and 10–890 µg Cu/l for *Tubifex* sp (Flemming and Trevors 1989). *C. riparius* proved the most sensitive species to Cu (LC_{50} 0.043 mg/l) when compared to *H. azteca* and *T. tubifex* (Milani *et al.* 2003), *Chironomus decorus* had a 48 h LC_{50} of 0.74 mg Cu/l (Kosalwat and Knight 1987). The LC_{50} 48 h for 2nd instar of *Hydropsyche angustipennis* was as low as 2.5 µg Cu/l (Geest *et al.* 1999). LC_{50} values for freshwater oligochaetes exposed to Cu were reported as 2.3 (24 h) to 0.9 (96 h) mg Cu/l for *Nais* sp. (Rehwoldt *et al.* 1973) and 0.34 mg Cu /l for *T. tubifex* after 48 h exposure (Rathore and Khangarot 2002). Even though Cu seems to disrupt ion balance and disturbs fish migratory behavior at > 4 µg/l, *T. tubifex* proved very tolerant to Cu with an LC_{50} after 24 h higher than that of benthic chironomids.

Ni was not toxic for *T. tubifex* up to 100 mg Ni/l; concentration-dependent effects were found neither for behavior nor for survival. This tolerance was higher than previously reported in the literature. Rathore and Khangarot (2002) reported an LC_{50} 96 h at 15^0C for *T. tubifex* of 10 mg Ni/l, Rehwoldt *et al.* (1973) reported an 24 h LC_{50} for *Nais* sp as 4.6 mg Ni/l. A large range of sensitivity for Ni was found for 12 ciliate species, ranging from 24 h LC_{50} values of 0.17 to 7.7 mg/l (Madoni 2000). Ni affected the filtration rate of the freshwater mussel *Dreissena polymorpha*, at an EC_{50} 48 h of 1.126 mg/l (Stuijfzand *et al.* 1995). The 96 h LC_{50} in goldfish was established at 80 mg Ni/l and at sublethal concentrations (25–75 mg/l) locomotory activity of goldfish was decreased (hypoactivity) (Ellgaard *et al.* 1995).

To the author's knowledge, there are no published studies on the effects of Ivermectin on aquatic invertebrates.

As the solvent methanol affected the locomotion of *T. tubifex*, tests with Ivermectin at concentration levels that need solvent addition (here 5 and 10 mg/l) are not regarded as ecologically relevant. Imidacloprid is considered as moderately toxic to aquatic fauna, *e.g.,* 48 h EC_{50} for immobility of *Daphnia* sp. was 0.09 mg/l, the 48 h LC_{50} for *Aedes aegypti* was 0.045 mg/l (Song *et al.* 1997). In the present study, *T. tubifex* had a 24 h LC_{50} of 0.3 mg/l for Imidacloprid, whereas Högger and Ammon (1994) reported an LC_{50} of 0.02 mg Imidacloprid/l for *T. tubifex*.

T. tubifex proved to be a tolerant organism. Behavioral responses showed high EC_{50} values compared to those reported for behavioral responses in aquatic invertebrates exposed to metals. For example, Cd exposure caused a decrease in locomotory activity of mayflies, and increased activity of fish (Riddell *et al.* 2005), but showed no difference in burrowing behavior of mayflies at concentrations of 0.02 mg Cd/g DW (Gosselin and Hare 2004). For Cu the EC_{50} value was much lower than the LC_{50} value, thus locomotory behavior was a more sensitive test parameter than survival in *T. tubifex*. This is supported by Lopes *et al*. (2004), who found that avoidance behavior of *Daphnia longispina* in a Cu-gradient was much more sensitive than survival as toxicity parameter (Lopes *et al*. 2004). Moreover, the 5th instar of *H. angustipennis* showed an EC_{50} 48 h for ventilation behavior at 0.017 mg Cu/l, and for inactivity 0.16 mg Cu/l (Geest *et al.* 1999), while the EC_{50} 24 h of locomotory behavior in *T. tubifex* was 3.8 mg/l in the present study. Cu was found not to affect caudal regeneration behavior in *T. tubifex* (Bouche *et al.* 1999a). Previous work suggested that *T. tubifex* might tolerate metal contamination by accumulating metals in the caudal region followed by subsequent automaty (Bouche *et al.* 1999b); however, even though Cd induced autotomy, no clear differences in Cd concentration levels between anterior and posterior parts were found (Bouche *et al.* 2003).

Also organic contaminants affect behavior, such as TCBP causing a decrease in burrowing behavior and feeding behavior of *Lumbriculus variegatus* (Landrum *et al.* 2004a,b). In the present study the EC_{50} 24 h values for Ivermectin and Imidacloprid were much lower than those for the essential metals Cu and Ni. For Imidacloprid the EC_{50} was lower than the LC_{50} after 24 h of exposure, thus locomotion represented a more sensitive test parameter than survival, as for the metals Cd and Cu.

CONCLUSIONS

The behavioral screening test for recording short-term toxic effects automatically and on a real-time basis proved to be practical even in a very tolerant test species. Behavioral effects could be seen before lethal effects. *T. tubifex* is a very tolerant test species for aquatic toxicity testing. The increasing order of toxicity for behavior was: Ni < Cu < Ivermectin < Cd < Imidacloprid, whereas for survival Cd and Ivermectin changed the ranking order. Responses in locomotory behavior (recorded as a decrease in locomotion) occurred at sublethal levels for Cu, Cd, and Imidacloprid, thus behavior was more sensitive than survival as a test parameter.

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Figure 1. Different types of behaviors in groups of *T. tubifex* in an MFB screenshot:
Left side: original locomotion signal (y: Volt, x: time), right side: Fast Fourier Transformation (FFT)-histogram, (y: occurrence of signal frequencies (%), x: frequency (Hz)).
Locomotion (above), aggregation of groups of worms showing synchronous identical ventilation behavior (middle, bottom) with a typical frequency of 1- 1.5 Hz.

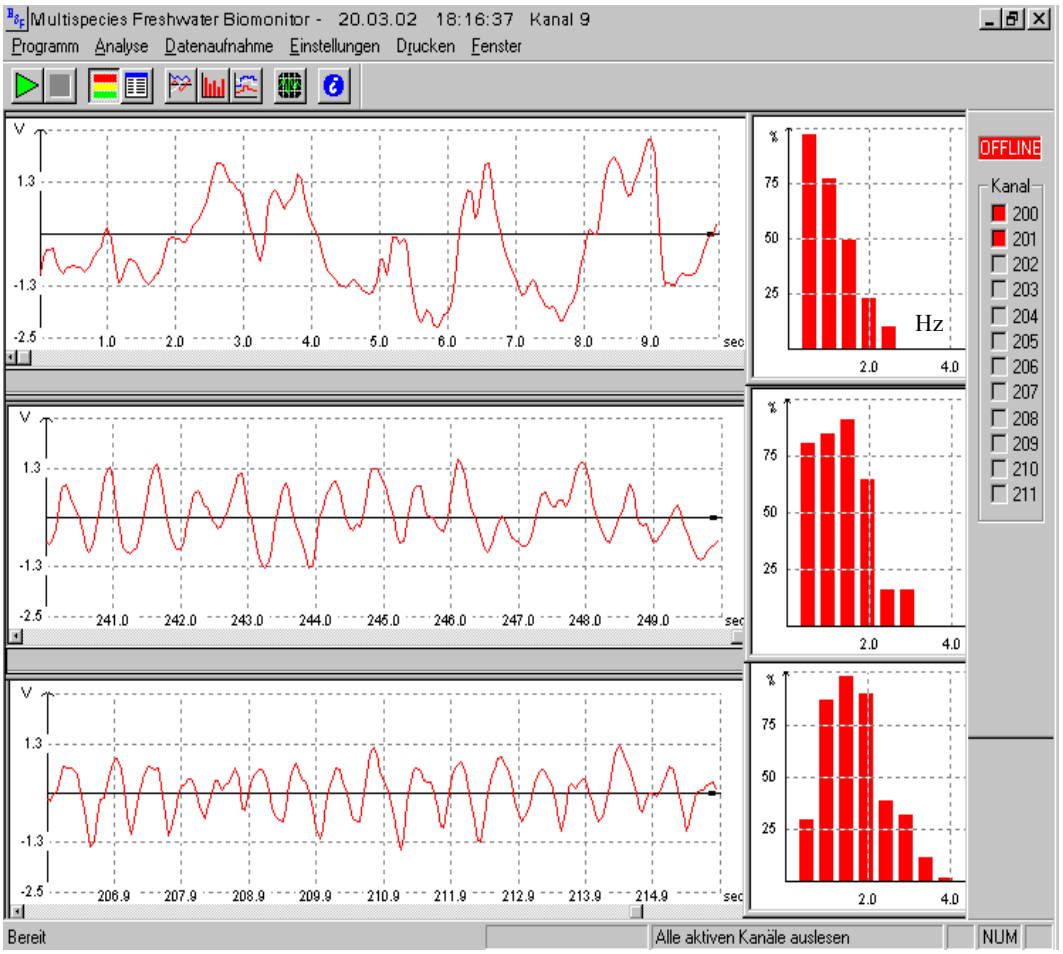

Table 1. **Survival**: A. Equations of linear regressions (x = log(concentration+1), y = probit of survival %) for the calculation of 24 h-LC_{50} for *Tubifex tubifex* for toxicants (for abbreviations, see text). B. Statistics, one factor ANOVA on arcsine $[(survival\%/100)]^{0,05}$ with concentrations as treatments and post-hoc Tukey's test. p< 0.05 *, p< 0.01 **, p< 0.001 *** .

A

Substance	**Equation**	**24h-LC_{50} (C.I. 95 %) mg. l^{-1}**	***r²***	***p***
Ni	y = 6.57-0.003x	-	-	ns
Cu	y = 8.57-286x	15.22 (12.49-20.38)	0.66	***
Cd	y = 9.44-5.71x	4.89 (3.90-5.92)	0.90	**
IV	y = 6.81-3.99x	1.82 (1.51-2.39)	0.39	***
ICP	y = 7.25-19.05x	0.32 (0.23-0.48)	0.56	***

B

Substance	***F(df₁, df₂)***	***p***	**Significant treatment (mgl^{-1}) ranking (Tukey's test: p<0.05)**
Ni	-	ns	-
Cu	$F(6, 17) = 35.96$	***	5<10<0.05<0.1 and 0.01 and 0
Cd	$F(7, 14) = 470.48$	***	10 and 5 < all other treatments
IV	$F(6, 23) = 7.69$	***	10<1<5<0.1<solvent control and 0.5 and 0
ICP	$F(F(8, 15) = 45.12$	***	10 and 1<0.1< all other treatments

IV: Ivermectin, ICP: Imidacloprid

Table 2. Linear regressions between locomotory activity (L) of *Tubifex tubifex* and concentrations (C) of toxicant, and in a three-dimensional plane as a function of concentration and time (ti).

Pollutant	Time (ti)	Equation	r^2	*p*
Nickel	0-24 h	L = 39.34+0.05C+0.18T-0.11(CxT)	0.25	-
	0-6 h	L = 39.20-0.01C	0.001	ns
	6-12 h	L = 40.20-0.22C	0.15	***
	12-18 h	L = 40.60-0.32C	0.31	***
	18-24 h	L = 39.82-0.35C	0.41	***
Cadmium	0-24 h	L = 32.89-1.24C-5.30T-0.07(CxT)	0.20	-
	0-6 h	L = 29.91-1.27C	0.06	*
	6-12 h	L = 19.64-1.44C	0.07	**
	12-18 h	L = 15.09-1.46C	0.09	**
	18-24 h	L = 13.7-1.48C	0.09	**
Copper	0-24 h	L = 40.86-1.13C-8.38T-0.18(CxT)	0.29	-
	0-6 h	L = 36.83-1.39C	0.06	**
	6-12 h	L = 19.18-1.61C	0.10	**
	12-18 h	L = 12.49-1.17C	0.08	**
	18-24 h	L = 12.42-1,.8C	0.03	ns
Ivermectin	0-24 h	L = 47.36-2.87C-9.11T+0.24(CXT)	0.4	-
	0-6 h	L = 42.25-2.72C	0.32	***
	6-12 h	L = 23.35-2.40C	0.23	***
	12-18 h	L = 18.58-1.73C	0.15	***
	18-24 h	L = 14.06-2.17C	0.07	*
Imidacloprid	0-24 h	L = 51.00-104.63C-2.56T-41.30(CxT)	0.20	-
	0-6 h	L = 46.29-126.61C	0.05	ns
	6-12 h	L = 49.20-227.04C	0.25	***
	12-18 h	L = 43.87-219.01C	0.21	***
	18-24 h	L = 39.25-262.88C	0.20	***

-: 3d nonlinear regression gives no p values

Table 3. Statistics on the locomotion data (x in %) of *Tubifex tubifex* during 24 h. Repeated measures ANOVA on arcsine$(x/100)^{0.05}$ transformed data averaged per time period of 6 h (4 time blocks, t_1-t_4) (R = 3, N = 3). Significant treatments (conc. in mg/l, time as nr. of time block) are ranked according to post hoc Tukey's test.

Pollutant	Concentration effects $F(df_1, df_2)$ p	Time effects (t_{1-4}) $F(df_1, df_2)$ p	Conc. x Time term $F(df_1, df_2)$ p
Cadmium	$F(7, 16) = 2.6$ $p = 0.05$ 10<others	$F(3, 48) = 185.3$ $p<0.0001$ t_1, t_2>t_3>t_4	$F(21, 48) = 2.3$ $p = 0.008$
Copper	$F(8, 18) = 2.5$ $p = 0.03$ only within t_3: c>others	$F(3, 48) = 290.9$ $p<0.0001$ t_1>t_2>t_3>t_4	$F(21, 48) = 2.4$ $p = 0.005$
Nickel	$F(7, 8) = 3.4$ $p = 0.05$ 100<others	$F(3, 24) = 14.6$ $p<0.0001$ t_1>others	$F(21, 24) = 4.4$ $p = 0.0003$
Ivermectin	$F(4, 10) = 10.8$ $p = 0.001$ control, 0.1, 0.5>1, 5*, 10*	$F(3, 30) = 55.5$ $p<0.0001$ t_1>t_2>t_3>t_4	n.s.
Imidacloprid	$F(6, 10) = 9.0$ $p = 0.001$ 1<others	$F(3, 30) = 14.6$ $p<0.0001$ t_1, t_2>t_3>t_4	n.s.

*including solvent

Table 4. Linear regressions (p<0,001) for the calculation of EC_{50} for the locomotion (concentration at which time spent on locomotion is 50 % of the control) (locomotion = average of movements between 0,5-3,0 Hz) of *Tubifex tubifex* (R: 6-8 chambers with 25 organisms in each), measured with the MFB during 24 h every 10 min per track of 4 min (*i.e.,* 6 h = 36 data values per treatment). The EC_{50}s are given after 6, 12, 18 and 24 h exposure time to single contaminants.

Pollutant	Exposure	Regression equation locom. % of control = a+b.log(conc.+1)	r^2	EC_{50} ± 95% C.I. (mg/l)
Copper	6 h	y = 95.1-33.9x	0.14	20.4 (18.5 -21.4)
	12 h	y = 89.9-50.9x	0.20	5.2 (4.8-5.3)
	18 h	y = 86.8-54.4x	0.18	3.9 (3.8-4.2)
	24 h	y = 86.3-53.7x	0.17	3.8 (3.7-4.1)
Cadmium	6 h	y = 75.5-37.3x	0.14	3.7 (3.5-4.1)
	12 h	y = 70.0-45.1x	0.17	1.7 (1.5-2.0)
	18 h	y = 67.6-50.2x	0.19	1.3 (1.1-1.4)
	24 h	y = 67.2-51.0x	0.19	1.1 (0.9-1.2)
Nickel	6 h	n.s.	-	-
	12 h	least squares fit*	-	181.0*
	18 h	least squares fit	-	99.0
	24 h	least squares fit	-	86.0
ICP	6 h	y = 98.1-884.8x	0.32	0.14 (0.13-0.14)
	12 h	y = 95.8-1032x	0.41	0.11 (0.10-0.12)
	18 h	y = 96.6-1215x	0.48	0.09 (0.08-0.10)
	24 h	y = 97.9-1358x	0.49	0.09 (0.08-0.10)
Ivermectin	6 h	y = 93.6-53.6x	0.43	5.4 (5.0-6.1)
	12 h	y = 86.1-67.2x	0.43	2.4 (2.2-2.5)
	18 h	y = 82.1-67.8x	0.37	1.8 (1.6-2.0)
	24 h	y = 81.9-68.5	0.37	2.0 (1.9-2.2)

* least square fit: visual estimate