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Can mouth part deformities of *Chironomus riparius* serve as indicators for water and sediment pollution? A laboratory approach

Miriam Langer-Jaesrich · Heinz-R. Köhler · Almut Gerhardt

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Abstract
Purpose The significance of chironomids mouthpart deformities as suitable indicators for pollutant contamination of natural waters and sediments has been investigated and discussed for several decades. Uncertainties still exist as further laboratory studies, with different pollutants and with the same experimental design are required.
Materials and methods In this study, the effects of four substances (i.e., nickel chloride, chlorpyrifos, imidacloprid and thiacloprid) were tested on the mouthpart deformity rates and patterns in *Chironomus riparius*. These compounds were investigated either individually or in mixtures.
Results and discussion No significant increase in the frequency of mouthpart deformities was found using different single substance treatments when compared to the controls. Consequently no concentration–effect relationships between substance concentration and deformity frequency were detected. In mixture experiments an increase in mouthpart deformities of *C. riparius* exposed to imidacloprid–thiacloprid mixtures was detected. This indicated that the effects of single substances and mixtures on mouthpart deformity frequency may differ considerably.
Conclusions The findings in this study from different laboratory approaches in combination with the published literature questions the reliability of chironomids mouthpart deformities as indicators of freshwater and sediment contamination by toxic substances.

Keywords Chironomids · Mixture effects · Mouthpart deformities · Pollutant indicator

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M. Langer-Jaesrich (✉) · H.-R. Köhler
Animal Physiological Ecology Department,
University of Tübingen,
Konrad-Adenauer-Str. 20,
72072 Tübingen, Germany
e-mail: langermiriam@gmx.de

A. Gerhardt
LimCo International,
An der Aa 5,
49477 Ibbenbüren, Germany

1 Introduction

Mouthpart deformities of chironomids have been discussed as potentially suitable indicators for in situ bioassessment and biomonitoring of contamination stress in streams and lakes for decades (Bird 1994; Gerhardt et al. 2006; Groenendijk et al. 1998; Janssens De Bisthoven et al. 1998a; Meregalli et al. 2000; Warwick 1990). In numerous field studies a higher rate of chironomids with deformed mouthparts could be detected in polluted water bodies (Dermott 1991; Groenendijk et al. 1998; Janssens De Bisthoven et al. 1998a; Warwick 1990; Wiederholm 1984), possible reasons for these include contamination with radionuclides, oil compounds, Polycyclic aromatic hydrocarbons (PAH's), organochlorine pesticides, Polychlorinated biphenyls (PCBs) and especially heavy metals (Janssens de Bisthoven et al. 1998b). Although often the stressors and the increased frequency of mouthpart deformities coincide spatially, a number of criticisms need to be made, as follows: (1) When an increased frequency of mouthpart deformities occurs, some studies fail to find a correlation between toxicants and the deformities (Bird 1994; Dermott 1991; Jeyasingham and Ling 1997; Jeyasingham and Ling 2000; Nazarova et al. 2004; Reynolds and Ferrington 2001). (2) Due to the fact that many of the mentioned compounds have been found simultaneously in situ, no direct relationships could be established in most cases. (3) In some

unpolluted areas an increased rate of chironomids with deformed mouthparts could be found which varied seasonally (Jeyasingham and Ling 2000). Several studies suggest that the season does have a significant impact on the frequency of mouthpart deformities (Jeyasingham and Ling 1997; Reynolds and Ferrington 2001; Servia et al. 2000; Urk et al. 1992). These results complicate, even after the long time of discussion, the usage of chironomid deformities as pollutant indicators. Therefore, several questions remain to be addressed in further laboratory studies.

The mechanisms by which the deformities are induced by pollutants are not yet well understood. To date the issue whether mouthpart deformities develop in the endocrine-regulated moulting phase and if the disruption of this complex process is likely to occur at the basis of their ontogeny (Meregalli and Ollevier 2001) has not been resolved. Several studies have been conducted with endocrine disruptors which might induce deformities via interrupting the endocrine-regulated moulting process. With β-sitosterol (5–500 µg/L) and 17α-ethynylestradiol (1–10 µg/L) no significant induction of deformation frequency was found, whereas in the presence of 4-*n*-nonylphenol (10–100 µg/L), lower 17α-ethynylestradiol concentrations (10 ng/L) and bisphenol A (10 ng/L) a significant increase in deformation frequency was induced (Meregalli and Ollevier 2001; Meregalli et al. 2001; Vermeulen et al. 2000a; Watts et al. 2003). Nonlinear concentration induction pattern has been observed before with endocrine disruptors and invertebrates (Matthiessen 2008; Oehlmann et al. 2000; Welshons et al. 2003), but more information is needed to understand the mechanism of the induction of mouthpart deformations in chironomids.

Additionally, field observations of polluted sites and the increased frequency of severe deformities could not easily be reproduced in laboratory experiments. Even in the cases were the examined pollutants were able to induce an elevated deformity rate, in many studies a linear exposure–response relationship between contaminants and deformities has yet to be found (Dias et al. 2008; Martinez et al. 2001; Vermeulen et al. 2000a). These observations are not reliable to support the rational application of chironomids deformities as pollutant indicators, especially when other stressors in the field might also generate deformities in chironomids.

In most published laboratory studies, just a single stressor, in most cases a heavy metal, was used to induce mouthpart deformities in chironomids (Bird et al. 1995; Janssens de Bisthoven et al. 2001; Janssens de Bisthoven et al. 1998b; Martinez et al. 2001; Vermeulen et al. 2000a). However, polluted areas are usually contaminated by more than just a single substance. Reviews on this subject emphasise the need to provide more consistent background data on the casual agents of chironomid deformation (Vermeulen et al. 2000a). Because no standardised protocol exists, comparison of published results in terms of causal agents of deformation is limited (Vermeulen et al. 2000a).

Therefore, the aim of this study was to investigate *Chironomus riparius* deformities after lifetime exposure to different kinds of pollutants using the same experimental design. Larvae of *C. riparius* were exposed to stressors with different modes of action. The organisms were exposed to single substances as well as binary mixtures. In the first set of experiments, the toxic metal nickel with its unspecific mode of action (affects protein integrity and function) and the widely used neurotoxic insecticide chlorpyrifos, which is acting as an acetylcholinesterase inhibitor, were chosen. In the second set of experiments, the pesticides imidacloprid and thiacloprid, both exhibiting the same mode of action as agonists of the nicotinic acetylcholine receptor, were tested in single and mixed exposures. The rationale for the choice of the selected mixture combinations is that effect information from different mixture scenarios is needed. In the first combination (nickel and chlorpyrifos) the substances aim at different target sides and show different mode of actions, whereas in the second combination (imidacloprid and thiacloprid) the two substances have the same target side and mode of action.

The specific questions addressed in this study are:

1) Can substances with different modes of action induce mouthpart deformities in *C. riparius* at environmentally relevant concentrations?
2) Is there a concentration–response relationship between the substance concentration and the incidence of deformities?
3) Do single substances induce the same reactions, as when applied in mixtures?

2 Material and methods

Stock cultures of *C. riparius*, from different genetic sources, in order to avoid genetic impoverishment, (LimCo International, Germany; University of Joensuu, Finland and Universidade de Coimbra, Portugal), were kept as larvae in fine quartz sand and dechlorinated tap water under constant aeration. Every day the chironomid larvae were fed with finely grounded fish flakes (50% Tetramin, 50% Tetraphyll, Tetra, Germany). Dechlorinated tap water was exchanged one or two times per week. Before emergence occurred, a breeding cage (55×65×120 cm) was installed over the stock containers, in which the adults were allowed to fly, swarm and breed. The egg masses which were attached to the vessel wall were collected every morning and used subsequently for experiments. The stock breeding and all

experiments were conducted in a climatised chamber at 21.0±0.5°C, with a light-dark cycle of 16:8 h artificial daylight (Philips standard daylight 54765, 2500 lumen, Germany).

After oviposition, eggs of *C. riparius* were exposed to different nominal concentrations of the toxic metal Nickel (II)chloride hexahydrate ($NiCl_2$*$6H_2O$, CAS 7791-20-0, Roth, 98.0%), and the insecticides chlorpyrifos (CAS 2921-88-2, Riedel de Haën, 99.2%), imidacloprid (CAS 138261-41-3, Riedel de Haën, 99.9%), thiacloprid (CAS 111988-49-9, Riedel de Haën, 99.9%) and also to their combination (for detailed list of tested concentrations see Tables 1 and 2). Control treatments were conducted with dechlorinated tap water. Mixtures were prepared using the toxic unit concept with a two-ratio design. In the first ratio the toxic unit level was dominated by chemical 1 (TU-level 2/3 chemical 2, 1/3 chemical 2), whereas the second ratio the TU-level was dominated by chemical 2 (TU-level 1/3 chemical 1, 2/3 chemical 2).

Three or four replicate experiments were performed for each concentration level. After 3 days, 30 larvae in the first larval stage (L1) were transferred to 250 ml glass beakers containing dechlorinated tap water spiked with the respective toxic substance and spiked quartz sediments (particle size 0.1–0.3 mm), which were burned for 3 h at 500°C (Dehner, Germany). For spiking, 1 day before larvae introduction the sediment was covered with 200 ml of the respective test solution and was subsequently shaken for 24 h under exclusion of light. Chironomids were fed every day with powdered ground fish flakes (1:1 Tetraphyll, Tetramin) providing a food supply of ≥0.36 mg/day/larvae. Surviving larvae were weekly transferred to new beakers containing freshly spiked sand and test solution. Glass beakers were gently swayed until the larvae came to the surface and the remaining sand was rinsed in water to find residual larvae. Larvae that did neither move nor respond to stimulation with a pipette were considered dead. The mortality rate and behavioural activity was recorded during the procedure of sand exchange for the third instar larvae (L3) and for the fourth instar larvae (L4), 10 and 17 days, respectively, after oviposition. Number of emerged *C. riparius* and 'time till emergence' was recorded. After the chironomids started to emerge, the remaining exuviae of L4 larva with head capsules were collected and stored in 100% ethanol. Since not a hundred per cent of the exuviae could be recovered the number of examined mouthparts cannot be equalised to the number of emerged chironomids. One day prior to the morphological preparation, the head capsules were separated mechanically from the rest of the exuviae and stored in Rotihistol overnight (Carl Roth GmbH, Germany). The next day, the head capsules were mounted on a glass slide in Roti-Histokit (Carl Roth GmbH, Germany) with the ventral side

Table 1 Data from exposure experiments with nickel and/or chlorpyrifos

Substance 1: Nickel [µg/L]	Substance 2: chlorpyrifos [µg/L]	No. of replicates	No. of examined individuals	Total deformities [%]	Missing teeth [%]	Extra teeth [%]	Mentum split medial teeth [%]	Köhn gap [%]
0		3	56	7.14	7.14	0	0	0
100.00		3	38	15.79	13.16	0	0	2.63
1,000.00		3	66	7.58	7.58	0	0	0
2,500.00		3	13	0	0	0	0	0
5,000.00		3	5	0	0	0	0	0
10, 000.00		3	0	-	-	-	-	-
	0	3	51	7.84	5.88	0	1.96	0
	1.00	3	15	6.67	6.67	0	0	0
	5.00	3	0	-	-	-	-	-
	10.00	3	0	-	-	-	-	-
	50.00	3	0	-	-	-	-	-
0	0	6	143	9.79	9.09	0.70	0	0
130.00	0.033	3	24	12.50	8.33	4.17	0	0
130.00	0.13	3	33	3.03	0	0	0	0
270.00	0.066	3	13	15.38	0	0	15.38	0
330.00	0.33	3	46	8.70	0	0	0	0
670.00	0.165	3	51	0	0	0	0	0
1,340.00	0.33	3	20	10.00	0	0	0	0
2,000.00	10	3	23	-	-	-	-	-

The numbers of examined *C. riparius* mouthparts, the frequency of total deformities as well as the percentage of the different deformity types are displayed. The exposure lasted over the entire span of larval life (28 day)

Table 2 Data from exposure experiments with thiacloprid and/or imidacloprid

Substance 1: Thiacloprid [µg/L]	Substance 2: Imidacloprid [µg/L]	rep	No examined N	Total deformities [%]	Missing teeth [%]	Extra teeth [%]	Mentum split medial teeth [%]	Köhn gap [%]
0		4	89	16.85	8.99	6.74	1.12	0
0.10		4	89	19.10	16.85	3.37	0	0
0.50		4	74	9.46	2.70	5.41	1.35	0
1.00		4	65	18.46	6.15	1.54	13.31	0
2.50		4	0	-	-	-	-	-
5.00		4	0	-	-	-	-	-
10.00		4	0	-	-	-	-	-
	0	4	97	12.37	0	9.28	3.09	0
	0.10	4	85	9.41	1.18	3.53	4.71	0
	0.50	4	83	18.07	4.82	9.64	3.61	0
	1.00	4	27	11.11	0	7,41	3.70	0
	2.50	4	0	-	-	-	-	-
	5.00	4	0	-	-	-	-	-
	10.00	4	0	-	-	-	-	-
0	0	8	191	15.70	10.47	4.71	1.05	0.52
0.08	0.33	4	55	20.00	1.82	16.36	1.82	0
0.83	0.83	4	70	27.14	0	4.29	**22.86**	0
0.17	0.66	4	59	22.03	6.78	3.39	**15.56**	0
0.17	0.17	4	36	19.44	11.11	8.33	0	0
0.33	0.33	4	52	11.54	3.85	5.77	1.92	0
0.42	1.66	4	54	**33.00**	0	3.70	**33.33**	0
0.83	3.33	4	0	-	-	-	-	-
1.66	1.66	4	0	-	-	-	-	-
1.66	6.67	4	0	-	-	-	-	-

The numbers of examined mouthparts from *C. riparius*, the frequency of total deformities as well as the percentage of different deformity types are displayed. The exposure lasted over the entire span of larval life. Significant differences to the control treatments are indicated in bold face (Wilcoxon test $p \leq 0.05$)

up, then covered with a glass cover slip and squeezed gently. The strongly sclerotinised mentum and mandibles were evaluated through observation with a light microscope at 40× magnification (Zeiss Axiostar plus). Missing teeth, extra teeth, mentum split medial teeth and Köhn gaps were counted as deformities (Bird 1994; Gerhardt and Bisthoven 1995; Servia et al. 1998). Special care was taken to distinguish deformities from physical wearing. The ratio of individuals with deformed mouthparts to the total number of examined individuals was calculated (Hämäläinen 1999). Two approaches were used for the interpretation of calculated ratios. Firstly, the total deformity rate was calculated using the total number of individuals with deformed mouthparts to the total of examined individuals. Secondly, the different deformity types were set in relation to the total number of examined individuals.

Due to the non-normal distribution of some data sets, all data were analysed using non-parametric statistics. Significance was tested using Friedman's analysis of variance (Statistica 5.0; Statsoft, USA), followed by a Wilcoxon two-group test (JMP 4.0, SAS systems; USA) to examine differences between control and exposure treatments.

3 Results

In all control and exposure treatments, deformities of the mandibles and the mentum did occur at rates between 0% and 33% (see Tables 1 and 2). In most cases, minor deformities such as missing or extra teeth occurred. Severe deformities (Köhn gaps) were detected only in two cases (at different treatments). Missing teeth in the mentum or the mandibles were the most abundant types of deformities found.

In the treatment with increasing nickel chloride concentrations (e.g., 100, 1,000, 2,500 and 5,000 µg NiCl/L) no significant differences were found between the control and the different nickel treatments, neither in the total deformity rates nor in the rates of the specific deformations (see Table 1). Consequently, no concentration–response relationship could be detected. No significant difference was

observed in the total deformity or specific deformity rates between the control treatments and the lowest chlorpyrifos concentration (1 µg/L). At higher chlorpyrifos concentrations, no *C. riparius* individuals survived into the pupal stage (following L4 larvae stage), therefore, no L4 exuviae were available for mouth deformity analysis. For this reason, in the mixture experiments low chlorpyrifos concentrations were combined with those of nickel chloride (see Table 1). In these mixtures, no significant differences in the total or specific deformity rates between the control treatments and the different mixture levels could be detected. Thus, from these experiments, no evidence for a dose–response relationship between the stressors and the deformity rate was found.

Also in the thiacloprid treatment (0.1, 0.5 and 1 µg/L) no significant difference in the total or a specific deformity rate between the control and the different thiacloprid treatments was found (see Table 2). At higher thiacloprid concentrations *C. riparius* larvae died before reaching the pupal stage. Therefore no mouthparts were available for analysis at high toxic substance concentrations. Also, in the imidacloprid treatments with 0.1, 0.5 and 1 µg/L, no significant difference in the total or specific deformity rates between the control treatments and the different thiacloprid levels was detected (see Table 2). At higher imidacloprid concentrations, *C riparius* larvae died again before reaching the pupal stage.

In binary mixtures of thiacloprid and imidacloprid, a significant increase of larvae with mentum split medial teeth was found in three experimental conditions: (1) 0.83 µg/L thiacloprid–0.83 µg/L imidacloprid; (2) 0.416 µg/L thiacloprid–1.66 µg/L imidacloprid; (3) 0.166 µg/L thiacloprid–0.66 µg/L imidacloprid (see Table 2 and Fig. 1). The pattern of mentum split and medial teeth formation could be best explained by assuming a model of concentration addition (Jonker et al. 2005). It was noted that in all thiacloprid–imidacloprid mixtures high percentages of deformities occurred, whereas this was not observed when organisms were exposed to single pesticide conditions at sublethal concentrations.

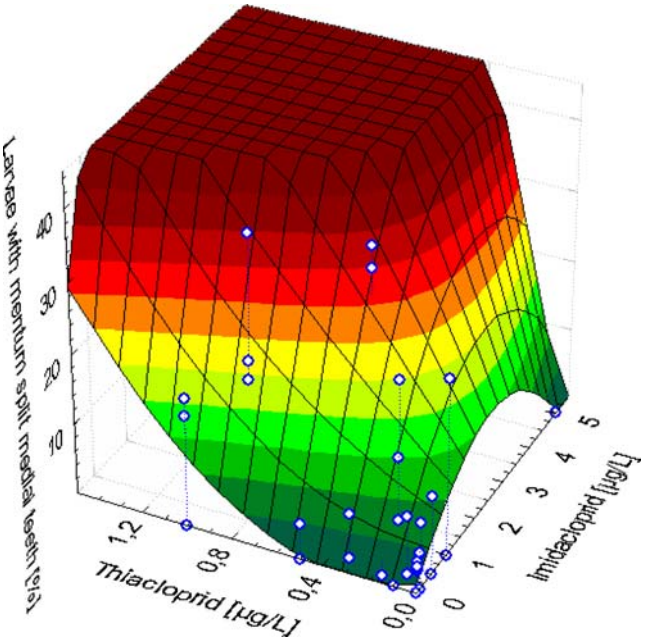

Fig. 1 Three-dimensional diagram (surface plot with *isobolic lines* calculated on the basis of means) of the number of *C. riparius* larvae with mentum split medial teeth after being exposed to different concentrations of thiacloprid and/or imidacloprid during the whole larval stage

4 Discussion

In this study, a high total deformity rate (between 7% and 17%, mean±SD: 11.37%±4.14%) was found in the control treatments. This high percentage of mouthpart deformities in the control is not unusual for *C. riparius*, a chironomid species which naturally shows a high percentage of deformities (Reynolds and Ferrington 2001; Servia et al. 2000). In other laboratory studies the deformity rate in the controls ranged between 7% and 34.1% (Janssens de Bisthoven et al. 1998b; Meregalli and Ollevier 2001; Meregalli et al. 2001; Vermeulen et al. 2000a). As a possible explanation they assumed that the high deformity rate in the controls had been due to a high degree of inbreeding in a laboratory culture. In this study, this factor was tried to be avoided, as the founding individuals of our culture originated from three different stocks of *C. riparius*. Additionally, the cultures were refreshed with new genetic material regularly. It has been suspected before that filter tissue used as substrate in some assays (Meregalli et al. 2001) or diatomaceous earth may impact the frequency of deformity in chironomids. Therefore, in this experiment, quartz sediment, free of possible organic contaminations, was used.

Until now, in most laboratory studies, only the effects of single substances of similar chemical properties (particularly heavy metals) on mentum deformities have been studied. The experimental designs varied substantially among most studies and therefore, the results are difficult to relate to one another. In the present study, several pollutants with different modes of action were assessed, for the first time, using a similar experimental design. By testing a single substance, it is not possible to induce a significant increase in the rate of mouthpart deformities of L4 larvae at sublethal concentrations neither for nickel chloride (100–10,000 µg/L) nor for insecticide compounds, including chlorpyrifos (1–50 µg/L), thiacloprid (0.1–10 µg/L) or imidacloprid (0.1–10 µg/L).

Therefore, the first approach to induce mouthpart deformities in *C. riparius* with substances exhibiting different modes of action was not successful. Hence, it is

self-evident that also the second (establishing concentration–response relationship between of the substance concentration and deformity incidences) in this study could not be verified. The results of the present study are in good agreement with those of other studies which showed that significant increases in the frequency of mouthpart deformities could not be induced in chironomids by exposure to substances like azaarenes, 17 α-ethynylestradiol, lead, mercury and β-sisterol (Bleeker et al. 1999; Meregalli and Ollevier 2001; Vermeulen et al. 2000a). Additionally, a linear concentration response, which would simplify the quantification of pollutants in sediment or water, has not yet been found (Dias et al. 2008; Kwak and Lee 2005; Martinez et al. 2001; Martinez et al. 2003; Watts et al. 2003). As well, it would be very desirable to find a connection between a specific deformity pattern and a special pollutant for the further use of deformities as indicators of water contamination. Only two studies have provided evidence for such a correlation (Dickman and Rygiel 1996; Vermeulen et al. 1998). These studies found different mouthparts which seemed to react independently to pollution stress and which displayed specific deformation frequency profiles (Vermeulen et al. 1998). Even though we tested only a limited number of chemicals we did not observe such a corresponding pattern with specific substance exposure, as well as in other recent studies.

For nickel, the absence of deformities was unexpected due to the fact that this is a widely distributed heavy metal. Heavy metals have been investigated in connection with deformity induction, both in the laboratory and the field (Bird et al. 1995; Janssens de Bisthoven et al. 2001, 1998b; Vedamanikam and Shazili 2009), (Bhattacharyay et al. 2005; Bird et al. 1995; Dickman and Rygiel 1996; Gerhardt and Bisthoven 1995; Gerhardt et al. 2006; Groenendijk et al. 1998). Nickel is toxic to chironomids affecting their survival rate (LC_{50}—20 days: 200 μg/L), behavioural activity (lowest observed effect concentration (LOEC) 2,500 μg/L)) and their emergence rate (LOEC 2,500 μg/L; Gerhardt and Janssens de Bisthoven 2006). But although nickel in the tested concentration range is obviously toxic to *C. riparius*, no significant increase in the frequency of mouthpart deformation was found, proving mouthpart deformities to be a less sensitive parameter than, e.g., behaviour or emergence. The higher tested nickel concentrations are above the environmentally relevant levels, with an exception of severely contaminated sited, where nickel concentrations can range between 50 and 2,000 μg/L in waters near industrial sites and 183,000 μg/L near a nickel refinery (Chau and Kulikovsky-Cordeiro 1995; Kasprzak 1987).

The induction of mentum deformities by pesticides was investigated to a lesser extent (Fowlkes et al. 2003; Madden et al. 1992). Only one study (Madden et al. 1992) found a significant concentration–response relationship between DDT and abnormal mentum. In the present study no significant induction or dose response was found in the mouth deformity rate of *C. riparius* exposed to the neurotoxic insecticide chlorpyrifos. Chlorpyrifos is a broad-spectrum organophosphorus insecticide (Richardson 1995) which is one of the most common active compounds in commercial pest control products worldwide (Dow AgroSciences 2008). Environmental chlorpyrifos concentrations of 0.19–0.3 μg/L were detected in urban waterways in California and surface waters in the USA (Bailey et al. 2000; Gilliom et al. 2006). In sediments a maximum concentration of 924 μg/kg chlorpyrifos was detected after a single rainstorm event in the Lourens River, South Africa (Schulz 2001). The effects of chlorpyrifos on chironomids have been assessed in various studies (Ankley et al. 1994; Belden and Lydy 2000; Callaghan et al. 2001; Fisher et al. 2000; Lydy et al. 1999; Moore et al. 1998). However, mouthpart deformity quantification was not the objective of these works. As it was expected from previous study, chlorpyrifos was highly toxic to *C. riparius* affecting the survival rate (LOEC 5 μg/L; LC_{50}—16 days: 5 μg/L, LC_{50}—29 days: 2.5 μg/L). Only at 1 μg/L chlorpyrifos, the chironomids survived longer than 11 days. In this chase no impairment of behavioural activity, growth, emergence rate and mouthpart deformity rate could be observed (Gerhardt and Janssens de Bisthoven 2006).

When being exposed to the insecticides thiacloprid or imidacloprid, larvae of *C. riparius* did not show significant changes or concentration response in mouth deformity rates. Thiacloprid is highly toxic to *C. riparius* affecting the survival rate (LOEC 0.5 μg/L), behavioural activity (LOEC 1 μg/L), the relative Hsp70 level (1 μg/L) and the total emergence rate (L0EC 0.5 μg/L) (Langer-Jaesrich et al. 2009). Imidacloprid is slightly less toxic, affecting the survival rate (LOEC 1 μg/L), behavioural activity (LOEC 5 μg/L) and the total emergence rate (L0EC 1 μg/L; Langer-Jaesrich, unpublished data). Both substances are members of a very successful new insecticide group called neonicotinoids (Maienfisch et al. 2003), which have been the fastest growing class of insecticides in modern crop protection in recent years (Jeschke and Nauen 2008). Information about measured imidacloprid and thiacloprid concentrations in the environment are rare. However, in their pesticide surface water report, Pfeuffer and Matson (2001)—cited in Jemec et al. (2007)—mentioned imidacloprid concentrations between 1 and 14 μg/L in water systems, USA. In a study by Süß et al. (2006), thiacloprid concentrations of 4.5 μg/L were detected in a surface water system near apple orchards in the surroundings of Hamburg, Germany.

The number of examined mouthparts decreased due to an increasing mortality at higher concentration levels of the

toxic substances tested. Because of this, data concerning the induction of mouth deformation by a particular toxic substance condition should be considered with care. This was the reason why Vermeulen et al. (2000a) postulated that a standard protocol should yield an adequate number of larvae to been screened at the end of a toxicology assay. It is of equal importance to observe the induction of deformities over the whole toxic substance concentration range under otherwise identical conditions, in order to quantify concentration–response relationships. Therefore, Vermeulen et al. (2000a) did also postulate that wide range concentration gradients are needed to obtain significant regression equation parameters. Because of this large concentration range required, a reduced number of surviving larvae at higher concentrations of tested substances had to be accepted in this study.

Using the experimental approach described previously it was not possible to see whether the deformity rate of dead larvae was higher than that of surviving larvae, due to the fact that, even under laboratory conditions, the dead larvae disintegrated within 2 days. Under natural conditions it is likely that dead larvae even disintegrate much faster due to scavengers and microbiological activity. Therefore, it seems to be likely that under field conditions the most sensitive chironomids die and consequently, fewer and less sensitive animals remain to show deformities.

The use of exuviae from the L4 stage in this study led to the advantage that the chironomids could complete their life cycle. This made it possible to compare mouthpart deformity rates with other accepted endpoints like emergence rates and developmental times (OECD 2004a, b). In this study, it was found inappropriate to include the mouthparts from exuviae of younger larvae mainly because they were difficult to recover in reliable numbers. Additionally, Vermeulen et al. (2000b) observed that older larvae displayed a higher frequency of deformities than younger larvae. Most deformities in the Vermeulen study were transferred identically from the L3 to the L4 stadium in an identical shape during moulting. In a very small number of individuals it was shown that a few deformities did not occur again in the L4 stadium. This provides evidence that deformities can be reversible.

The third goal of this study was to test whether single substances are able to induce the same mouthpart deformations as induced by substance mixtures. This is a fundamental question concerning freshwater systems and sediments because many different kinds of pollutants are continuously being introduced into the environment. Several studies in the field have suggested that deformity rates of chironomids can be used as bioindicators for pollutants in aquatic systems (Madden et al. 1992; Meregalli et al. 2000; Servia et al. 1998). However, only single substance tests have been conducted in the laboratory to assess the impact of these substances in nature. In a single field study the concentrations of different toxic substances have been summed up (simulating toxicant additivity) and normalised to the content of organic matter and clay (Meregalli et al. 2000). However this application does not take into account synergistic or antagonistic deviations from the common concepts of concentration addition or independent action (Jonker et al. 2005) where a synergistic deviation would be a more toxic reaction than expected from the concentration addition concept and an antagonistic deviation would correspond to a less toxic reaction than the independent action concept predicts (Escher and Hermens 2002).

In the present study no significant change or dose response in mouth deformities of *C. riparius* exposed to mixtures of nickel and chlorpyrifos was found (see Table 1). The survival rate, however, was significantly decreased (Gerhardt and Janssens de Bisthoven 2006). Chironomids exposed to mixtures of imidacloprid and thiacloprid showed a partially increased rate of mouthpart deformities (e.g., mentum split medial teeth) when compared to the control treatments (see Table 2 and Fig. 1). This was unexpected since both substances exhibited the same mode of action and did not induce deformities in single substance exposure experiments, at the same total concentration range. These differences in mouthpart deformity induction after exposure to single substances or pollutant mixtures render unreliable the potential application of mouthpart deformities as bioindicators for pollutants.

In summary, the results of the present study show that not all toxicants (including heavy metals and pesticides) are able to induce mouthpart deformities in chironomids, even when these groups have been reported to correlate with these abnormalities (Bhattacharyay et al. 2005; Madden et al. 1992; Vermeulen et al. 2000a). Compared to mouthpart deformity rates other frequently tested endpoints such as activity changes, total emergence rate and survival rate were more sensitive and consistently showed a concentration-dependence for all of the substances (Gerhardt and Janssens de Bisthoven 2006; Langer-Jaesrich et al. 2009; Langer-Jaesrich, unpublished data). Because of this and the mentioned absence of inducible deformities in most of the experiments in this study, it has to be questioned whether mouthpart deformities reflect the health condition of *C. riparius* adequately and whether these abnormalities can be used as reliable bioindicators of pollution. Furthermore, the often observed nonlinear concentration response and the different response to mixtures and single substances hamper the application of the deformities as bioindicators under laboratory conditions.

In nature a different situation of even higher complexity may occur due to the fact that chironomids are exposed to pollutants over several generations and have the time to adapt (Postma and Davids 1995). Other additional stressors

and seasonally fluctuating stress might complicate the interpretation of deformity data in nature. In the few published multi-generation studies investigating deformity rates (Janssens de Bisthoven et al. 2001; Janssens de Bisthoven et al. 1998b), deformity rates fluctuate and complicate interpretations. Furthermore another challenge will be to extend the existing knowledge on whether mouthpart deformities in a given larval stage could induce similar deformities in the subsequent stage or whether they can be reversed by the moulting process (Vermeulen et al. 2000b).

The findings in this study form different laboratory approaches in combination with the published literature questions the reliability of chironomids mouthpart deformities as indicators of freshwater and sediment contamination by toxic substances.

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