Assessment of potential endocrine disrupting agrochemicals in freshwater systems: Full life-cycle testing over two generations with the aquatic midge *Chironomus riparius* (Meigen)

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If pesticide registration is to become more responsive to ecological issues, the information and approach to determining potential effects must be made explicitly ecological. The current focus is foremost on chemistry and toxicology.

Kapustka et al. (1996)

The current cumulative thesis is based on the following scientific publications:

I. Tassou KT, Schulz R. 2011. Two-generation effects of the chitin synthesis inhibitor, teflubenzuron, on the aquatic midge *Chironomus riparius*. Ecotoxicology and Environmental Safety 74:1203-1209.

II. Tassou KT, Schulz R. 2012. Low field-relevant tebufenozide concentrations affect reproduction in *Chironomus riparius* (Diptera: Chironomidae) in a long-term toxicity test. Environmental Science and Pollution Research. Accepted: 7 November. DOI 10.1007/s11356-012-1311-4.

III. Tassou KT, Schulz R. 2012. Combined effects of temperature and pyriproxyfen stress in a full life-cycle test with *Chironomus riparius* (Insecta). Environmental Toxicology and Chemistry 31:2384-2390.

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List of abbreviations

ANOVA	Analysis of Variance
ATSDR	Agency for Toxic Substances and Disease Registry
BPUs	Benzoylphenylurea
C	Chironomus
cm	Centimeter
CSI	Chitin Synthesis Inhibitor
°C	Degree Celsius
D	Daphnia
d	Days
DDT	Dichlorodiphenyltrichloroethane
DMF	Dimethylformamid
DMSO	Dimethylsulfoxid
dw	Dry Weight
EC	European Commission
EC_{10}	Concentration showing 10% Effect
EC_{50}	Median Effect Concentration
ED	Endocrine Disruption
EDC(s)	Endocrine Disrupting Chemical(s)
EDIETA	Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment
EEC	European Economic Community
EFSA	European Food and Safety Authority

EQR	Environmental Quality Standards
ERA	Ecological Risk Assessment
EU	European Union
g	Gravity
h	Hour
IGRs	Insect Growth Regulators
JHA(s)	Juvenile Hormone Analogue(s)
Kg	Kilogram
K_{ow}	Water:octanol partition coefficient
L	Liter
LC-MS	Liquid Chromatography Mass Spectrometry
LOEC	Lowest Observed Effect Concentration
lx	Lux
MAC	Maximum Allowable Concentration
min	minutes
mL	Milliliter
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-operation and Development
PCBs	Polychlorinated Biphenyls
PPP	Plant Protection Panel
REACH	Registration, Evaluation Authorization and Restriction of Chemicals
SEPA	Scottish Environment Protection Agency

SPE	Solid Phase Extraction
SUR	Sulfonylurea Receptor
TFB	Teflubenzuron
UDP-N	Acetylglucosamine 1- carboxyvinyltransferase
µg	Microgram
µg/kg	Microgram pro kilogram
µg/kg dw	Mikrogram pro kilogram dry weight
µL	Mucroliter
WFD	Water Framework Directive

Summary

Agricultural pesticides, especially insecticides, are an integral part of modern farming. However, these may often leave their target ecosystems and cause adverse effects in non-target, especially freshwater ecosystems, leading to their deterioration. In this thesis, the focus will be on Insect Growth Regulators (IGRs) that can in many ways cause disruption of the endocrine system of invertebrates. Freshwater invertebrates play important ecological, economic and medical roles, and disruption of their endocrine systems may be crucial, considering the important role hormones play in the developmental and reproductive processes in organisms.

Although Endocrine Disruption Chemicals (EDCs) can affect moulting, behaviour, morphology, sexual maturity, time to first brood, egg development time, brood size (fecundity), and sex determination in invertebrates, there is currently no agreement upon how to characterize and assess endocrine disruption (ED). Current traditional ecotoxicity tests for Ecological Risk Assessment (ERA) show limitations on generating data at the population level that may be relevant for the assessment of EDCs, which effects may be sublethal, latent and persist for several generations of species (transgenerational). It is therefore the primary objective of this thesis to use a test method to investigate adverse effects of EDCs on endpoints concerning development and reproduction in freshwater invertebrates. The full life-cycle test over two generations that includes all sensitive life stages of *C. riparius* (a sexual reproductive organism) allows an assessment of its reproduction and should be suitable for the investigation of long-term toxicity of EDCs in freshwater invertebrates. *C. riparius* is appropriate for this purpose because of its short life cycle that enables the assessment of functional endpoints of the organism over several generations. Moreover, the chironomid life cycle consists of a complete metamorphosis controlled by a well-known endocrine mechanism and the endocrine system of insects has been most investigated in great detail among invertebrates. Hence, the full life–cycle test with *C. riparius* provides an approach to assess functional endpoints (e.g. reproduction, sex ratio) that are population-relevant as a useful amendment to the ERA of EDCs.

In the laboratory, *C. riparius* was exposed to environmentally-relevant concentrations of the selected IGRs in either spiked water or spiked sediment scenario over two subsequent generations. The results reported in this thesis revealed significant effects of the IGRs on the

development and the reproduction of *C. riparius* with the second (F1) generation showing greater sensitivity. These findings indicated for the first time the suitability of multigenerational testing for various groups of EDCs and strongly suggested considering the full life-cycle of *C. riparius* as an appropriate test method for a better *assessment* of EDCs in the freshwater environment.

In conclusion, this thesis helps to detect additional information that can be extrapolated at population level and, thus, might contribute to better protection of freshwater ecosystems against the risks of Endocrine Disrupting Chemicals (EDCs.) It may furthermore contribute to changes in the ERA process that are necessary for a real implementation of the new European chemical legislation, REACH (Registration, Evaluation Authorization and Restriction of Chemicals). Finally, significant interactions between temperature, chemical exposure and generation were reported for the first time and, may help predict impacts that may occur in the future, in the field, under predicted climate change scenarios.

1 Introduction

1.1 Background information and definition of endocrine disruption

The endocrine system, along with the nervous system, is an integrating system that endogenously regulates the normal functions of other systems and maintains development in the face of a constantly changing environment (Flynn 2011; Marty et al. 2011). There is, however, evidence that a wide variety of compounds introduced into the environment by humans, may have the potential to interfere with the normal function of the endocrine system of humans and wildlife. The action of these so called xenobiotics may lead to the impairment of the homeostatic mechanisms of the organism or the initiation of processes at abnormal times in the life cycle. Disruption of the endocrine system can occur in various ways: by mimicking a natural hormone; over-responding to the stimulus; responding at inappropriate times; blocking receptor site of hormones on a cell; directly stimulating or inhibiting the endocrine system and causing overproduction or underproduction of hormones.

The most important evidence suggesting that exposure to environmental chemicals can cause disruption of endocrine function comes from many field observations on morphological and histological aspects made in a number of wildlife species. These effects have been linked to Endocrine Disruption (ED) and reported in mollusc (Albanis et al. 2006; Duft et al. 2005; Oehlmann and Schulte-Oehlmann 2003; Oehlmann et al. 2006; Schulte-Oehlmann et al. 2000), crustacea (Albanis et al. 2006; Baldwin et al. 2001; Mu and Leblanc 2002; Oda et al. 2005; Wang et al. 2005), insects (Albanis et al. 2006; ATSDR 2006; Boudjelida et al. 2005; Hahn et al. 2002; Smagghe et al. 2002; Watts et al. 2001), fish, reptiles, birds and mammals in various parts of the world. Though the issue of ED and potential effects of Endocrine Disrupting Chemicals (EDCs) on human health and wildlife has increasingly been attracting interest in all sectors of society (government, scientists, regulatory bodies, and industries), scientific findings and observations are often disputed among scientists, environmentalists, and authorities. This controversial discussion is mainly based on the definition of the ED and also on how the effects observed can without any doubt be related to the disruption of the endocrine system of wildlife. This latter concern is very important considering invertebrates, whose endocrine system is not completely understood as in vertebrates.

This thesis focuses on the ED in aquatic invertebrates and uses the adopted definition of the European Scientific and Regulatory Community as the reference to develop a reliable test

design for hazards identification and characterization of potential endocrine disruption agrochemicals. This definition from the Weybridge Conference (Weybridge 1996, EC 1996) stipulates: “An endocrine disruptor is an exogenous substance or mixture that alters function (s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations”. Furthermore, “a potential endocrine disruptor is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism”.

The following groups of endocrine disruptors, i.e. compounds with hormonal activity, were identified by the EU-Strategy on EDCs (EC 1999):

Natural hormones from any animal, released into the environment, and chemicals produced by one species that exert hormonal actions on other animals, e.g. human hormones unintentionally reactivated during the discharge of human waste in sewage effluent may result in changes to fish.

Natural chemicals including toxins produced by components of plants (the so-called phytoestrogens, such as genistein or coumestrol) and certain fungi.

Synthetically produced pharmaceuticals that are intended to be highly hormonally active, e.g. the contraceptive pill and treatments for hormone-responsive cancers, may also be detected in sewage effluent.

Man-made chemicals and by-products released into the environment. Laboratory experiments have suggested that some man-made chemicals might be able to cause endocrine changes. These include some pesticides (e.g. DDT and other chlorinated compounds), chemicals in some consumer and medical products (e.g. some plastic additives), and a number of industrial chemicals (e.g. polychlorinated biphenols or PCBs, dioxins). The hormonal activity of these chemicals is many times weaker than the body's own naturally present hormones, e.g. nonyl phenol (a breakdown product of alkylphenol ethoxylate surfactants), found as a low level contaminant in some rivers in Europe, has an oestrogenic activity only about one-ten thousandth that of the natural hormone, oestrogen.

This latter group of compounds intentionally introduced into the environment by human activity is the main topic of this thesis. The focus is on the chronic exposure effects of

pesticides that are suspected to have the potential to interfere with the endocrine system in freshwater invertebrates.

1.2 Endocrine disruption in invertebrates

The issue of ED in aquatic invertebrates has not attracted as much interest in the literature as aquatic vertebrates until the workshop in the Netherlands (1998) on Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment (EDIETA) (deFur et al. 1999), where the importance of invertebrates in assessing ED was pointed out. Moreover, there are many important factors which determine the study of invertebrates in ecotoxicology at both the population and ecosystem levels. Invertebrates represent more than 95% of all known species in the animal kingdom (Wilson et al. 1999), and are much more abundant than vertebrates. They are present in nearly all types of ecosystems. They constitute a very important part of the global biodiversity with key species for the structure and function of aquatic and terrestrial ecosystems (Oetken et al. 2004) and represent the primary animal source in the food web (Gourmelon and Ahtiainen 2007). They often play a key role in different food chains, determining interrelationships and participating in upward biomagnification of chemicals in the food web. They are present in all heterotrophic layers, utilizing a variety of food, and take part in the decomposition of organic matter, transfer of biogenic substances and xenobiotics (Migula 2005). In addition, deFur (2004) suggested that the invertebrate EDC assays may be useful in predicting or indicating potential EDC responses in vertebrates. The author reported that in this capacity, invertebrate assays may serve either as sentinels of potential effects from exposure to conditions or chemicals, or as predictors of effects that have a counterpart in other or many species.

Considering the above-mentioned importance and utility of invertebrates, disturbances in their development and reproduction might therefore be of concern to the biological diversity and function of ecosystems. It is therefore important to take this biodiversity into account when addressing the potential adverse effects caused on developmental and reproductive parameters by EDCs. In Europe, the ecological monitoring including invertebrate assessments is a key part of the Water Framework Directive (WFD) (Allan et al. 2006).

Due to the important ecological role that insects play in freshwater ecosystems, ED in aquatic insects is evident and a matter of some concern. Indeed, among invertebrates, the endocrine system of insects has been most intensively investigated (de Fur et al. 1999; Soin and

Smagghe 2007; Taenzler et al. 2007). The endocrinological information available on insects provided an advantage over other classes of invertebrates in the assessment of EDCs. This requires an understanding of the endocrine system of test species (Soin and Smagghe 2007) to assess unambiguously an endocrine disrupting effect. Several ecologically relevant insect species with short life cycles are available, which enable assessment over several generations for persistent compounds and especially EDCs. Due to the development of the 3rd generation insecticides (juvenile hormone (ant) agonists or ecdysone (ant) agonists)-targeting the endocrine system of the target species (agricultural pests and vectors of disease), the issue of assessing the effects of these pesticides on non-target freshwater insects is a necessity for the ecosystem health.

1.2.1 **Assessment of endocrine disrupting effects in aquatic invertebrates: Full life-cycle over two generations**

OECD test guidelines recommend using aquatic invertebrates for assessing the effects of chemicals on the reproductive output tests at organism, population and community levels for legislative purposes of new or existing chemicals. Insecticides acting as hormone (ant) agonists may have adverse effects on non-target aquatic invertebrates (Taenzler et al. 2007). Furthermore, EDCs can affect moulting, behaviour, morphology, sexual maturity, time to first brood, egg development time, brood size (fecundity), and sex determination in invertebrates (OECD 2006).

Despite this wide spectrum of effects of EDCs, there is currently no agreement on how to characterize and assess ED. Traditional ecotoxicity tests for Ecological Risk Assessment (ERA) have limitations when generating data at the population level (Kramer et al. 2011; Newman et al. 2006) that may be relevant for the assessment of EDCs (Clubbs and Brooks 2007). Assessing population level effects, especially of EDCs, may be more relevant for protecting the ecosystem than risk assessment of individual organisms that may be more relevant for the protection of endangered species. Therefore, present guidelines that employ toxicity tests based on acute or chronic exposure for a maximum of one generation of species may not adequately contribute to the risk assessment of EDCs. Effects of EDCs may be sublethal, latent and persist for several generations of species. Hence, development of standardized tests to cover these types of effects is required (Taenzler et al. 2007).

As suggested by Gourmelon and Ahtiainen (2007), it is also important to have reliable test methods to evaluate adverse effects of chemicals, including those identified as potential EDCs, on endpoints concerning developmental and reproductive effects in invertebrates. In this context, Ducrot et al. (2010) proposed the development of partial life-cycle experiments in order to assess the effects of EDCs on the freshwater gastropod *Lymnea stagnalis* after observing that long-term effects of EDCs on aquatic invertebrates remain difficult to see. Therefore, the full life-cycle test with the freshwater midge *C. riparius* over two generations as presented in this thesis might be relevant for assessing the long-term toxicity of pollutants such as EDCs in freshwater invertebrates. Kramer et al. (2011) also suggested that life-cycle studies may be more appropriate for assessing the long-term risks associated with the exposure of a population to pollutants. Considering that full life-cycle tests may include endpoints that allow the extrapolation of data at the population level as a result of exposure to potential EDCs, this thesis may contribute to the recommendations of the Plant Protection Panel (PPP) at the EFSA for revisions of the Directive 91/414/EEC (Taylor and Blake 2009). The importance of full life-cycle tests in generating additional information relevant at population level has been acknowledged by the adoption of the OECD guideline 233 that was designed to assess the effects of life-long exposure to chemicals. This life-long exposure can extend over generations of species in the context of EDCs. Raimondo et al. (2009) suggested that the evaluation of population level and multigenerational effects is particularly important in the risk assessment of EDCs, because adverse effects may not be evident during the first generation of exposure.

Multigenerational studies include all successive sensitive life stages of an organism and take the measurement of functional endpoints (e.g. reproduction, sex ratio) that are population relevant into account. This is relevant for the assessment of any endocrine toxicity that should be accounted for in the life-cycle response of an organism. Moreover, multigenerational studies may be a useful amendment to the present ERA guidelines for aquatic invertebrates because natural populations can be exposed to pollutants over several generations. Especially for EDCs whose effects may be spread over generations of species, test designs over more than one generation with environmentally-relevant concentrations of pesticides as presented in this thesis may contribute to reflect upon and accurately predict population level effects of aquatic invertebrates'exposure to EDCs. This multigenerational aspect in assessing EDCs in aquatic invertebrates may provide valuable information that provides a new insight into the

ERA of EDCs, such as the detection of interaction effects between treatment and generation (Tassou and Schulz 2012).

Hommen et al. (2010) remarked for terrestrial invertebrates that, though the current risk assessment for agrochemicals (EDCs included) might protect against long-term effects on populations and communities, no uniform principles defining the data requirements and decision criteria have been refined. This reflects also the current situation for the aquatic invertebrates in assessing EDCs. Moreover, Bars et al. (2011) reported that although the European legislation on plant protection products only supports the marketing and use of chemical products on the basis that they do not induce endocrine disruption in humans or non target species, there are currently no guidelines on how to identify and evaluate endocrine activity and disruption.

1.2.2 **The freshwater midge *Chiromonus riparius* as test species**

C. riparius used here for the full life-cycle test over two generations belongs to the family Chironomidae (true or non-biting midges) that belongs to the order Diptera. As a freshwater species, it covers a highly relevant aquatic exposure route for various agrochemicals (EDCs inclusive) and its short life cycle (28 days) is adequate for chronic testing over several generations. *C. riparius* is a sediment dwelling organism whose first life stages take place under water. Larvae ingest particles from the sediment and the detritus, as well as bacteria and algae (Rasmussen 1984). They undergo a complete hormonally-controlled metamorphosis consisting of the egg stage, four larval stages, pupa and imago. The adult's sex can easily be determined by its body structure, and the sexual reproduction in *C. riparius* is an advantage compared to the organisms often used in assessing impacts upon endocrine-controlled reproductive processes, such as *Daphnia magna* (Crustacean) with a parthenogenetic reproduction. Furthermore, *C. riparius* can be used in water or sediment exposure, which represents an advantage over tests limited to a single media or matrix since biological impacts on aquatic organisms can result through exposure via the water phase and/or the sediment. All these above-mentioned reasons make chironomids well-suited to the risk assessment of EDCs in a full life-cycle test.

1.2.3 **Agricultural pesticides as endocrine disruptors**

Agricultural pesticides, especially insecticides, are an integral part of modern farming. They are highly useful to the growing of crops, but their effects are less than desirable when they

leave the targets of the agricultural ecosystems. Any unintentional loss of pesticide is not only wasteful, but also reduces efficiency and increases costs to the user and the non-target environment (Falconer 1998). Pesticide pollution from agricultural practices is widely regarded as one of the greatest causes of contamination of surface waters. Many of the agrochemicals, e.g. insecticides used to control outbreaks of agricultural pests, have been intentionally designed to interact with the hormonal system of the target insects, acting as ecdysone agonists, antagonists, or juvenile hormone analogues, which are the key hormones (Kropp et al. 2004) in insects that are relevant to the evaluation of EDCs. These agrochemicals have the potential to affect the freshwater biological community and ecological functions once they reach the system. The WFD was adopted in 2000 in Europe in order to prevent and reduce pollution, protect the aquatic environment and promote sustainability of aquatic ecosystems' functions.

1.2.4 **The selected agrochemicals for study**

Insect growth regulators (IGRs) are often referred to as third generation insecticides, developed to intentionally mimic, block or otherwise interact with the hormone system of insects (Oetken et al. 2004). Among this group of insecticides, a selection of IGRs was used as test substances for the assessment of potential adverse effects in *C. riparius*: teflubenzuron, a Chitin Synthesis Inhibitor (CSI); tebufenozide an ecdysone agonist and pyriproxyfen, a Juvevile Hormone Analogue (JHA). Detailed information about the insecticides and the effects is provided in each of the appendices.

2 Aim of the thesis

The lack of information regarding potential endocrine effects of agrochemicals on aquatic insect species is largely attributable to limited number of suitable test systems. The primary objective of this thesis is to use a newly developed test design (full life-cycle test) to assess developmental and reproductive ED effects in non-target aquatic insects as an amendment to the framework of revisions for Directive 91/414/EEC. It also aims to contribute to approving the suitability of *C. riparius* as a representative species of freshwater insects for EDCs testing. In order to fulfill these objectives, the above mentioned IGRs were selected and investigated.

In Appendix I the investigation of adverse effects of the CSI teflubenzuron is presented. CSIs are benzoylphenylurea (BPUs) compounds that interfere with larval development, thus disturbing moulting and resulting in deformations in the cuticle (Reynolds, 1987). Adults maturing from CSI-exposed larvae were reported to have several sub-lethal physiological abnormalities that might ultimately diminish physical and reproductive capacities (Desneux et al. 2007). Despite this wide spectrum, Desneux et al. (2007) observed that only studies reporting direct effects (mortality or emergence inhibition) of CSIs exist in the literature. The long-term effects of these compounds on the surviving adults or the next generation of non-target organisms with their ecological implications at the population level remain less examined (Desneux et al. 2007). Therefore, the purpose of this study was to assess for the first time the effects of teflubenzuron using a two-generation test to check for accumulation or carry-over effects on development and reproduction of the non–target species *C. riparius*.

In Appendix II the study of the effects of the ecdysone agonist tebufenozide that mimics natural moulting hormone in insects is shown. Ecdysone agonists are benzoyl hydrazines which have been reported to act as agonists of the ecdysteroidal moulting hormone at the molecular level and, therefore, cause a variety of hormonal effects in insects and crustacean arthropods (Dhadialla et al. 1998). Tebufenozide may be specific to lepidopteran insects, but its frequent detection in surface water discharging from a fruit orchard (Süß et al. 2006), justifies its investigation in the present study. Moreover, although tebufenozide has been suspected to persist at rather low concentrations in the aquatic ecosystems (Süß et al. 2006), until now no study has assessed its effects on the reproduction of an aquatic insect or investigated its long-term effects on freshwater organisms over more than one generation. Hence, the aims of the study were to assess for the first time the effects of tebufenozide on the reproduction of an aquatic insect and to investigate long-term effects of its environmentally-relevant concentrations on life cycle parameters of *C. riparius* over two generations. This may complement the current risk assessment and contribute to the characterization and detection of EDCs endpoints in aquatic insects.

In Appendix III the effects of the JHA pyriproxyfen are presented. Juvenile hormones are terpenoids produced by the *corpora allata* that primarily regulate metamorphosis and reproduction in insects. Organisms, especially ectotherms, may experience different temperatures in the field, in the context of global climate change. Therefore, two temperature levels were used in contrast to the traditional risk assessment tests usually performed in the

laboratory at a constant standard temperature. Hence, this third appendix contributed to our understanding of how responses of experimental populations may vary across temperature gradients and different levels of chemical stressors during their lifespan. This study also highlighted the interactions between temperature change, chemical stressor and exposure over generations of species, which might be relevant for a better ERA of EDCs in ecotoxicology. Knowing how laboratory populations may behave due to the interaction effects of temperature and pesticide stress over generations might help predict effects that will occur in the natural environment.

A schematic chart with the rationale for the three groups of IGRs tested is showed below (Fig. 1). This thesis also contributes to underlining the importance of conducting multiple generational studies for assessing information, which might be helpful in the risk assessment of persistent and endocrine disruptive agrochemicals compared to the traditional guidelines for risk assessment presently used.

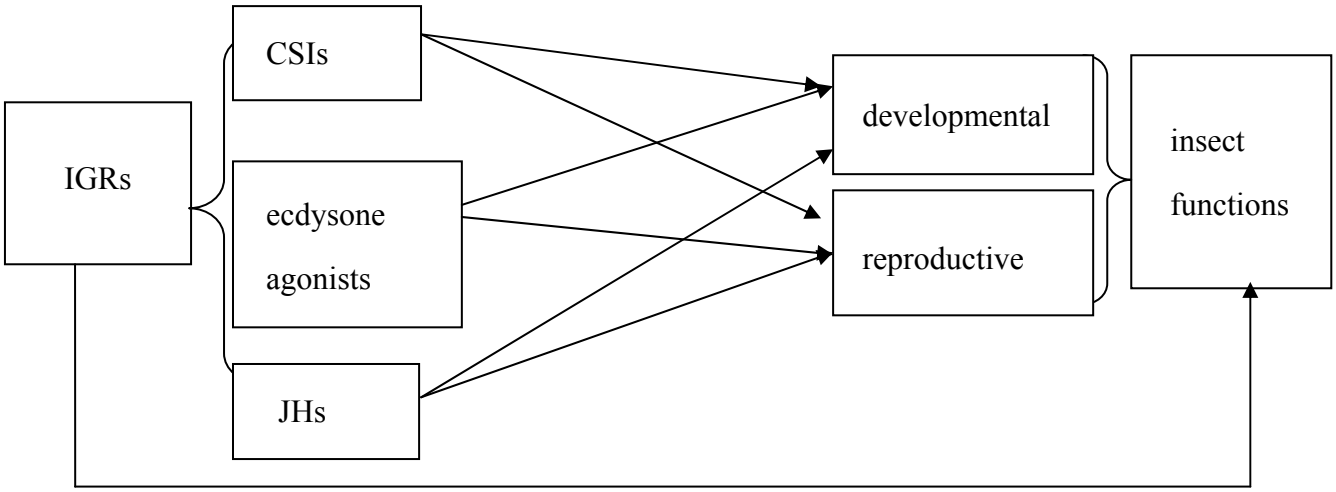

Fig.1 Schematic representation showing the rationale for the use of the different IGRs as test compounds for assessing effects on hormone-controlled functions in *C. riparius* over a full life-cycle test. Arrows indicate possible interference of each IGR in an insect's function. Each group of IGRs was investigated in each of the appendices of this thesis in order to evaluate additional population relevant information for a better risk assessment.

3 Test protocol

The method used in this thesis is designed for the exposure of *C. riparius* to the selected IGRs in either a spiked water or spiked sediment scenario, over two subsequent generations, in order to assess potential chronic sublethal effects. The test began with the exposure of first-instar larvae of *C. riparius* in test vessels covering all successive aquatic larval stages in the first (P) generation until emergence in the subsequent (F1) generation (Fig. 2). The larvae were exposed to five test concentrations of each of the test substances, including one solvent control, and fed on a daily basis with commercial fish-food (Tetra Min®). Eight replications of each treatment plus the solvent control were performed under static conditions. Observations were based on life cycle parameters of the emerged midges such as emergence ratio, sex ratio and, development rate. In each treatment, adult midges in the P generation were cautiously collected with an exhauster and transferred to two breeding cages (50 x 50 x 50 cm) for swarming, mating and oviposition into a 2-liter glass crystallising dish filled with the same sediment-water system as in test vessels. Besides the assessment of the reproduction (fecundity and fertility) in the P generation, fertile egg ropes at the peak of oviposition were selected in each breeding cage to start the F1 generation. After hatching, first-instar larvae within the same treatment were put together and randomly allocated to the newly prepared test vessels for observation until emergence. Compared to the standard *Chironomus* chronic test (OEDC 2004 a, b), the present design allowed the additional assessment of reproduction in the P generation as well as the F1 generation endpoints (emergence ratio, development rate, and sex ratio) to see if there is increased sensitivity of larvae owing to accumulation or carry-over effects. Detailed information on the test procedure with each selected IGR is given in the three appendices.

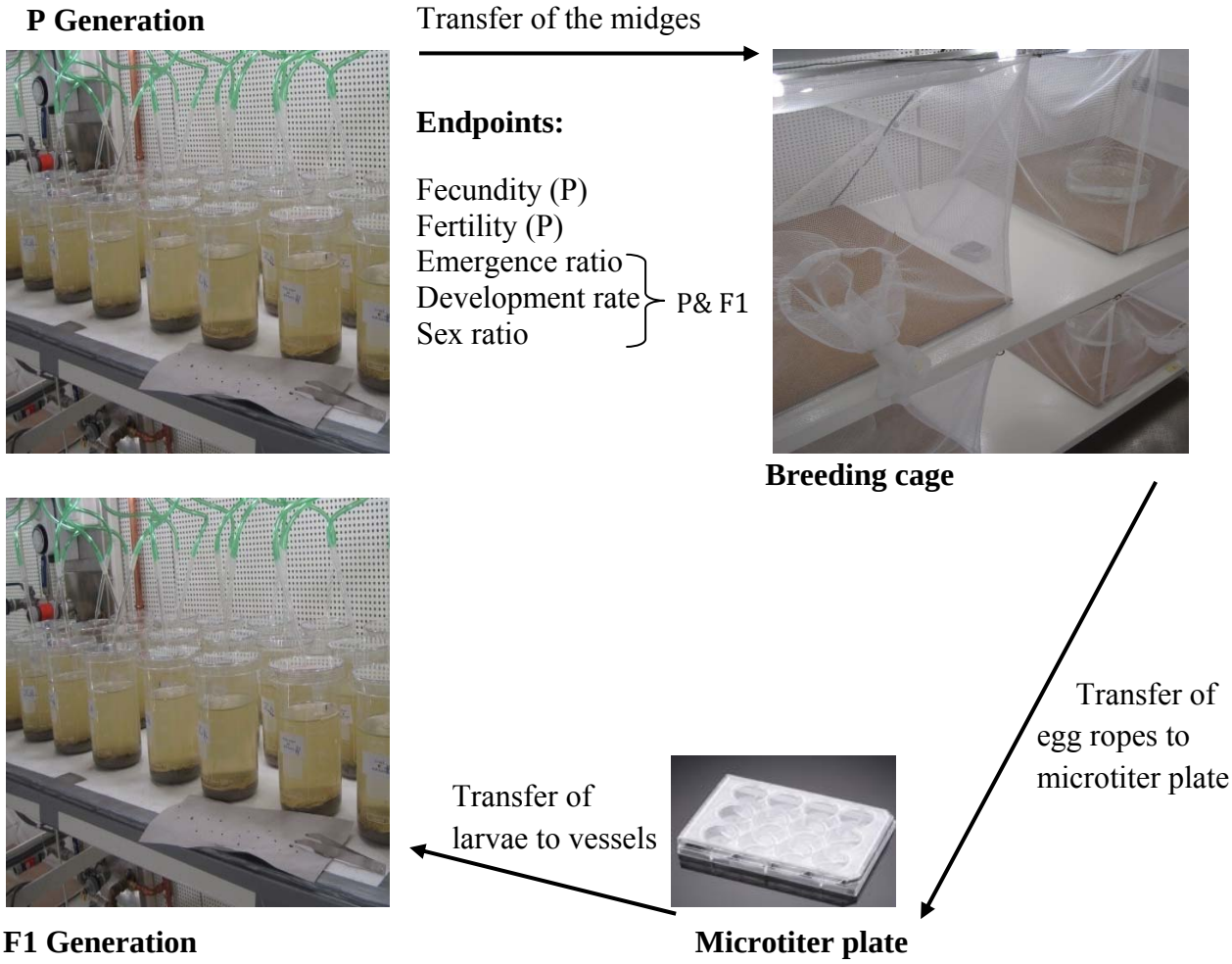

Fig. 2 Schematic presentation of the full life-cycle test with the freshwater midge *C. riparius*.The exposure to environmentally-relevant concentrations of the selected IGRs began with first-instar larvae of the P generation until emergence in the subsequent F1 generation.

4 Assessment of the selected IGRs

This thesis aims to assess the long-term effects of the selected IGRs on a non-target freshwater species for a better ecological hazard identification and characterization of these compounds. The results indicated that life cycle parameters of *C. riparius* were significantly affected as a consequence of the exposure over two generations to environmentally-relevant concentrations of the IGRs. Detailed information on the effects of each of the IGRs is provided in Appendices I to III. In addition, an overview of the effects on life cycle parameters of *C. riparius* after exposure to the different IGRs is provided below.

4.1 Assessment on the emergence ratio of the midges

A significant effect on the emergence ratio of *C. riparius* was observed in the present study with the second generation of midges showing more changes than the P generation for the CSI teflubenzuron [**Appendix I**] and the JHA pyriproxyfen [**Appendix II**], when compared to their respective solvent controls**.** A significant decrease in the emergence of *C. riparius* was observed in the P generation at the teflubenzuron concentration of 156.3 µg/kg dry weight of sediment (dw) compared to the solvent control [**Appendix I**]**.** In the F1 generation, a significant decrease in the emergence ratio (Dunnett's test $p < 0.001$) was observed at the test concentration of 100 µg/kg dw, indicating that the F1 generation showed more sensitivity than the P generation [**Appendix I**]. In addition, an increase in mortality (non- emerged midges) from 32% to 38% was observed at 100 µg/kg dw when the P generation was compared to the F1 generation.

The assessment of the JHA pyriproxyfen was made at different temperature levels (16 and 24°C) to give a scientific understanding of variable and suboptimal temperatures, which organisms, especially ectotherms, may experience in the field in contrast to tests for agrochemicals risk assessment that are still usually performed in the laboratory at a constant standard temperature.

At 16°C, a significant effect on the emergence ratio was observed at a pyriproxyfen concentration ≥ 10 µg/L in the P generation. At 30 µg/L, the emergence ratio showed a substantial deviation from the control, i.e., an emergence ratio reduced to zero (**Fig. 3).** In the F1 generation, the concentration of 1µg/L already produced a significant effect compared to the corresponding solvent control (**Fig. 3**). In addition, EC_{50}-values of 6.1 µg/L (95% CI, 3.2-11.3) and 1.2 µg/L (95% CI, 0.9-1.5) were calculated for the P and F1 generation respectively, indicating a five-fold increase in sensitivity in the F1 generation when compared to the P generation.

At 24°C, a significant decrease in the emergence ratio of midges was observed at 10 µg/L in the P generation (**Fig. 3**). In the F1 generation, the emergence ratio of the midges was not significantly affected in the concentration range of 1 to 3 µg/L. The EC_{50}-value for the P generation was 4.1µg/L (95% CI, 2.2-7.5). In the F1 generation, the EC_{50}-value was not calculated because the emergence ratio was over 86% in all treatments ranging up to 3µg/L.

A comparison of effects on the emergence ratio of *C. riparius* in the F1 generation at the chosen temperature levels showed more pronounced effects at 16°C than at 24°C (**Fig. 3**). However, the emergence time of the midges at 24°C was shorter compared to the emergence time at 16°C.

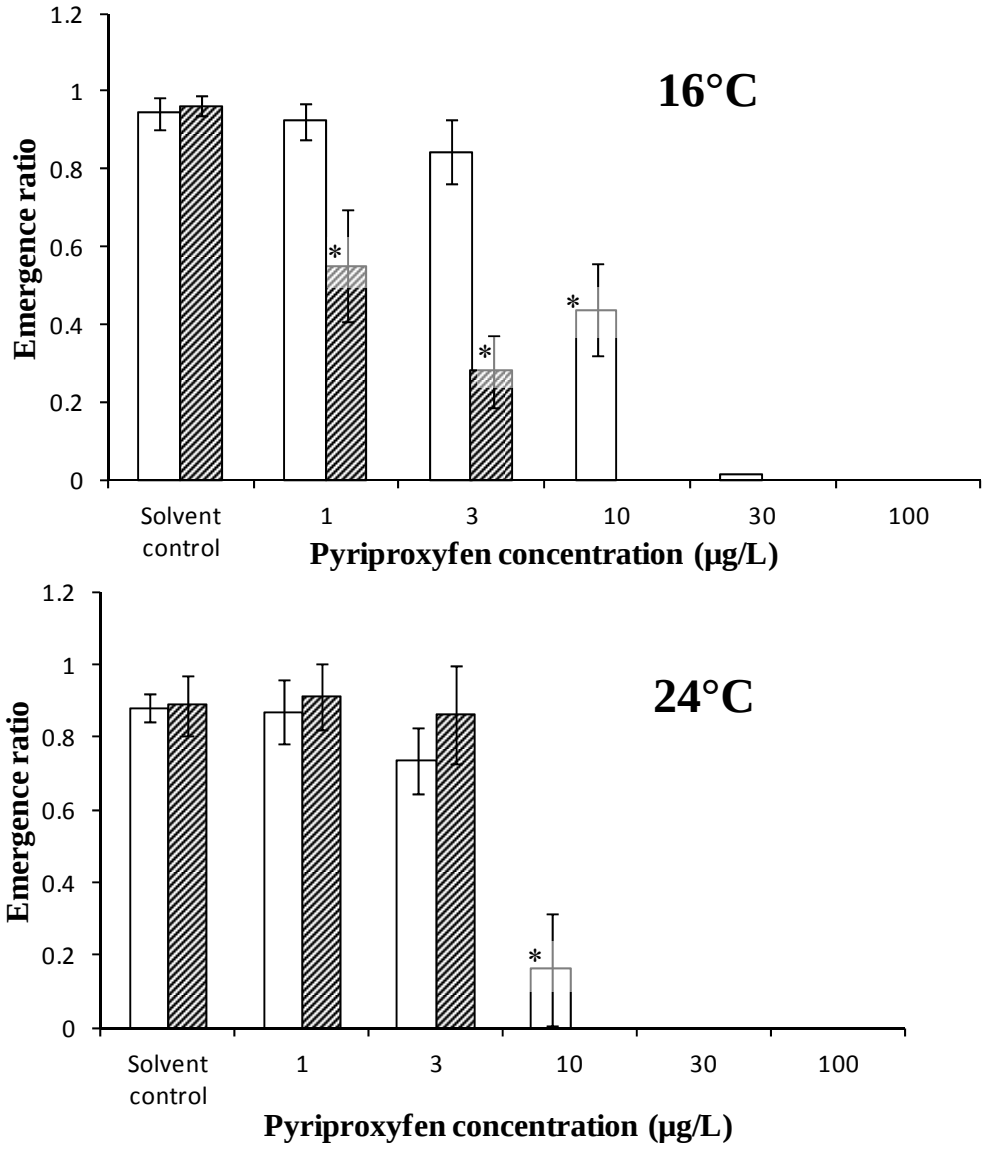

Fig. 3 Emergence ratio (±SD; n= 8) of *C. riparius* in the P generation (white bars) and F1 generation (hatched bars) at different temperatures during static exposure to pyriproxyfen. The asterisks denote significant differences (Dunnett's test) to the corresponding solvent control [see also **Appendix III]**.

A significant interaction effect of temperature and pyriproxyfen (factorial ANOVA, $p < 0.001$) was determined for the emergence ratio in the P and F1 generations. The present study also showed for the first time a significant three factorial interaction effect of temperature × chemical × generation ($p < 0.001$) on the emergence ratio of the midges (**Table 1**). This emphasized that, at 16°C, the strength of adverse effects of pyriproxyfen in the F1 generation was significantly higher than in the P generation.

For the ecdysone agonist tebufenozide, no significant decrease in the overall emergence ratio of midges was observed for the chosen concentration range of 4 µg/L-26.2 µg/L. However, the male'emergence ratio in both generations indicated different patterns (**Fig. 4**). The male fraction showed an increased level in the P generation without significance with increasing test concentrations. In the F1 generation, the male fraction was significantly elevated at 4 µg/L, and there was a trend to a decrease in the male fraction with increasing test concentrations (**Fig. 4**). Additionally, a significant two–way interaction of exposure × generation ($p < 0.004$) on male fraction was observed for the first time in the present full life-cycle test, while both exposure and generation individually did not indicate any significant effect (**Table 2**). These results demonstrated much lower limits for effects of tebufenozide in aquatic arthropods as reported until now in the literature, and showed that long-term tests with environmentally-relevant concentrations of EDCs may yield valuable additional information to improve upon the standard risk assessment.

Table 1: Factorial analysis of variance of the effect of temperature (16 vs. 24°C), chemical exposure (control vs. pyriproxyfen treatments) and generation (P vs. F1) on life parameters of *C. riparius* [see also **Appendix III]**.

Endpoint	**Source**	**df**	**MS**	**F**	**p**
Emergence ratio	Temperature	1	0.278	8.957	0.003
	Chemical	5	6.153	197.934	< 0.001
	Generation	1	0.261	8.387	0.004
	Temperature × Chemical	5	0.207	6.665	< 0.001
	Temperature × Generation	1	1.261	40.547	< 0.001
	Chemical × Generation	2	0.178	5.721	0.004
	Temperature × Chemical× Generation	2	0.414	13.305	< 0.001
Development rate	Temperature	1	0.021	2.527E3	< 0.001
	Chemical	3	0.000	19.313	< 0.001
	Generation	1	0.000	57.322	< 0.001
	Temperature × Chemical	3	2.668E-5	3.155	0.028
	Temperature ×Generation	1	2.574E-6	0.304	0.582
	Chemical × Generation	2	1.628E-6	0.193	0.825
	Temperature × Chemical× Generation	2	1.872E-5	2.213	0.115

df = degrees of freedom; MS = mean square; F = likelihood ratio; p = probability

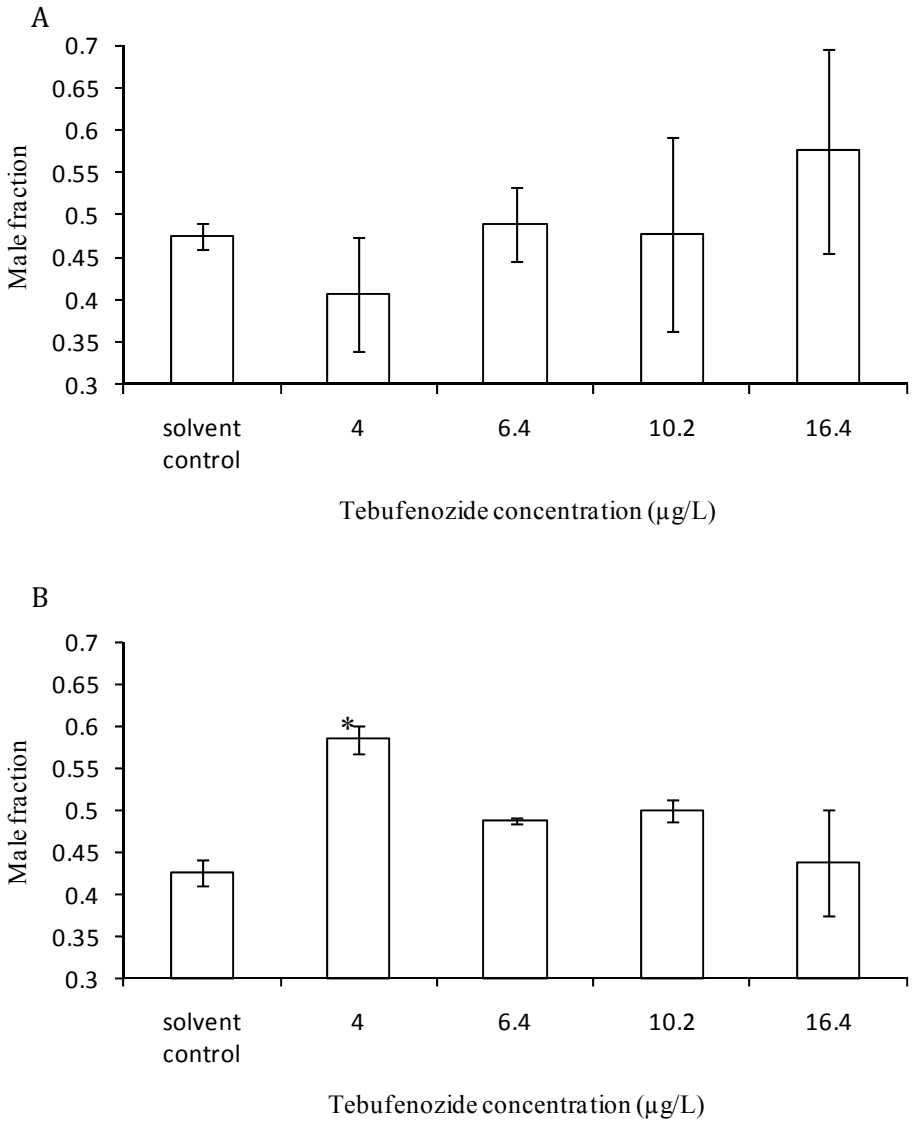

Fig. 4 Mean male fraction (± standard deviation, n = 8) of *C. riparius* after static exposure to tebufenozide over two generations (A: P generation; B: F1 generation). The asterisk denotes a significant difference compared to the solvent control (Fisher's Exact Test, p = 0.009) [see also **Appendix II]**.

Table 2: Factorial analysis of variance of the effect of exposure (control vs. tebufenozide treatments) and generation (P vs. F1) on the male fraction of *C. riparius* [see also **Appendix II**].

	Source	**df**	**MS**	**F**	**p**
Male fraction	Exposure	3	0.001	0.083	0.969
	Generation	1	0.004	0.311	0.579
	Exposure × Generation	3	0.066	4.935	0.004
	Error	56	0.013		

df = degrees of freedom; MS = mean square; F = likelihood ratio; p = probability

4.2 Assessment of the development rate of the midges

Significant adverse effects of teflubenzuron on the mean development rate of midges were observed at 100 µg/kg dw when compared to the solvent control (Dunnett's test $p < 0.001$) in the P generation. In the F1 generation, no effects on the mean development rate were observed in treatments ranging up to 100 µg/kg dw when compared to the solvent control [**Appendix I**]. No significant decrease in the overall mean development rate even at the highest concentration tested of 26.2 µg/L tebufenozide in the P and 16.4 µg/L tebufenzozide in the F1 generation was observed in the study. However, the male mean development rate showed an overall significantly lower value (paired sampled test $p < 0.001$) in the F1 generation compared to the P generation (**Fig. 5**).

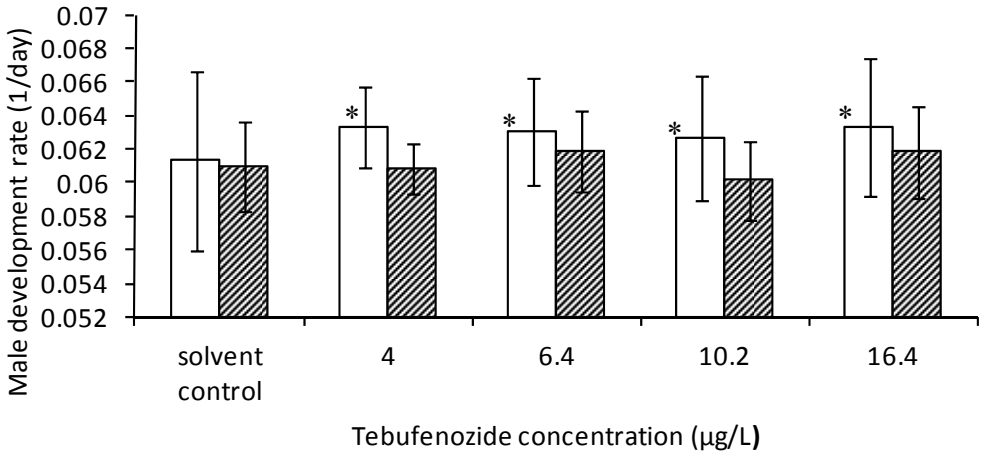

Fig. 5 Mean (± standard deviation, n = 8) development rate of males of *C. riparius* after static exposure to tebufenozide over two generations. The male development rate in the tebufenozide treatments of the P generation (white bars) was significantly higher (paired t-test; $p < 0.001$) than those of the F1 generation (hatched bars), denoted with asterisks [see also **Appendix II**].

As the assessment of pyriproxyfen was conducted at different temperature levels, the effect of temperature alone on the mean development rate of *C. riparius* was first analyzed in controls at each temperature. As expected, an elevated temperature considerably influenced the midges' mean development rate. In other words, the development time (as the reciprocal of the development rate) was shorter in the elevated temperature of 24°C than at 16°C (**Table 3**).

Table 3: Temperature effect on the mean development rate of controls of *C. riparius* during two generations at the temperature levels of 16 and 24°C.

Temperature regime	Development rate (1/day)	
	P generation	F1 generation
16°C	0.048	0.045
24°C	0.079	0.072

In addition, at both 16 and 24°C, a decrease in the mean development rate of control midges was observed in the F1 generation when compared to the P generation (**Table 3; Fig. 6**), indicating that the midges were probably still not fully acclimated to the selected temperatures.

In pyriproxyfen treatments, variations of response in the mean development rates among the different temperature regimes were observed. At 16°C, a significant decrease in the mean development rate was observed in the P generation at 3 µg/L compared to the corresponding solvent control. In the F1 generation, the mean development rate showed a significant reduction starting at 1 µg/L, underlining a greater sensitivity in the F1 generation (**Fig. 6**). EC_{10} estimation on the mean development rate of midges provided values of 2.98 µg/L (95% CI, 0.36-3.72) and 1.69 µg/L (95% CI, 0.78-2.60) for the P and F1 generation, respectively.

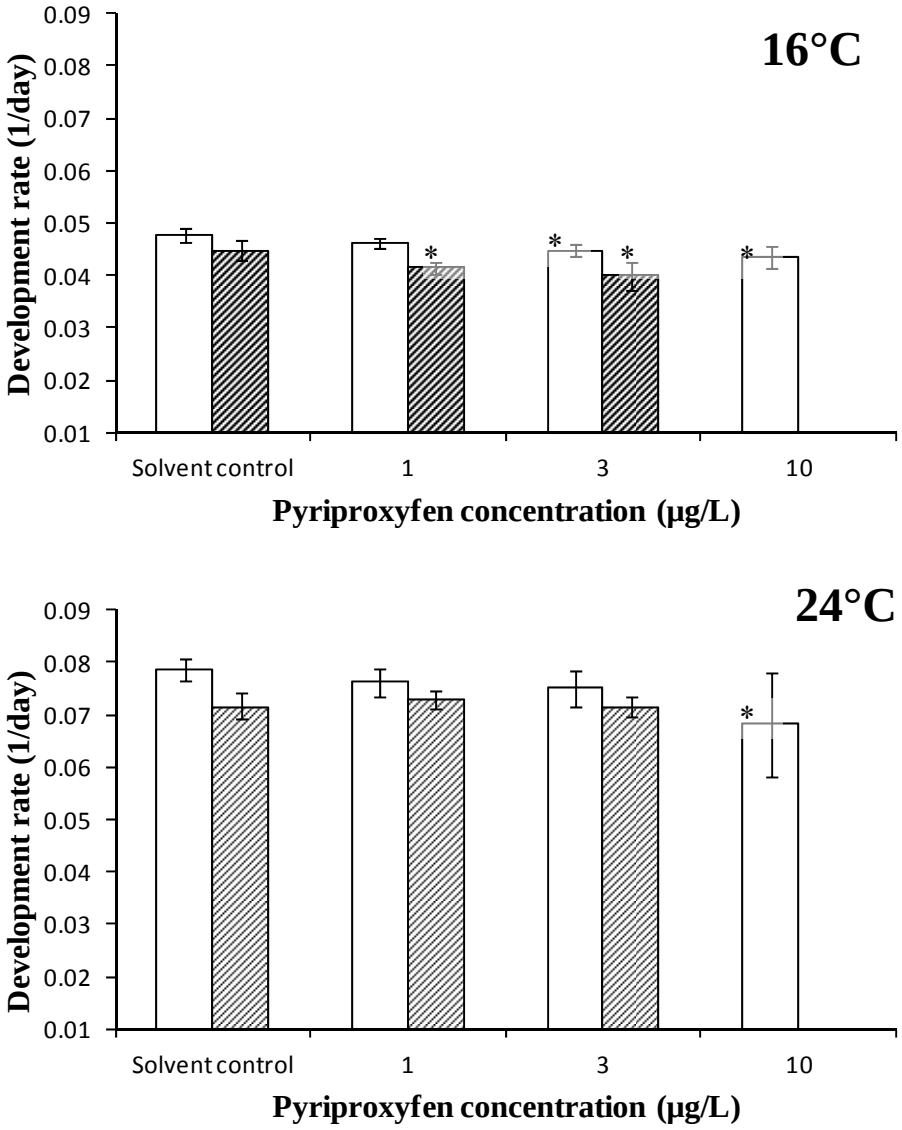

Fig. 6 Mean development rate (±SD; n= 8) of *C. riparius* at different temperatures in the P generation (white bars) and the F1 generation (hatched bars) with pyriproxyfen exposure. Asterisks denote significant differences (Dunnett's test) from the corresponding solvent control [see also **Appendix III]**.

In the P generation at 24°C, a significant effect on the mean development rate of midges was observed at 10 µg/L compared to the corresponding solvent control (**Fig. 6**). The F1 generation showed no significant effect on the midges' mean development rate at 1 µg/L and 3 µg/L pyriproxyfen. EC_{10} values of 2.77 µg/L (95% CI, 2.12-3.42) and 2.81µg/L (95% CI, 1.79-3.52) were calculated for the P and F1 generation, respectively.

No significant temperature × chemical interaction effect (p = 0.158) on the mean development rate in the P generation was determined while these combined factors significantly (p = 0.003) affected the F1 generation (**Table 4**). However, no significant temperature × pyriproxyfen × generation effect (p = 0.115) was determined on the mean development rate of the midges, though each of the stressors temperature, chemical and generation individually and significantly affected the development rate (**Table 1**). Significant combined effects of temperature × chemical (p = 0.028) on the mean development rate were observed but not for temperature × generation and chemical × generation (**Table 1**).

Table 4: Factorial analysis of variance of the effect of temperature (16 vs. 24°C) and chemical exposure (control vs. pyriproxyfen treatments) on the development rate of *C. riparius* for both parental (P) and filial (F1) generation [see also **Appendix III]**.

	Source	**df**	**MS**	**F**	**p**
Development rate (P)	Temperature	1	0.013	1.063E3	< 0.001
	Chemical	3	0.000	12.106	< 0.001
	Temperature × Chemical	3	2.156E-5	1.804	0.158
Development rate (F1)	Temperature	1	0.011	2.643E3	< 0.001
	Chemical	2	2.952E-5	7.304	0.002
	Temperature × Chemical	2	2.640E-5	6.531	0.003

df = degrees of freedom; MS = mean square; F = likelihood ratio; p = probability

4.3 Assessment of the reproduction of the midges

The assessment of the reproduction of *C. riparius* was conducted in the P generation and reflected significant effects of all the selected IGRs in this freshwater non-target species. Results of the CSI teflubenzuron indicated that at 156.3 µg/kg dw, no female was able to lay an egg rope (**Fig.7**); the concentration of 112.7 µg/kg dw (95% CI, 82.05 and 143.4 µg/kg dw) was calculated (Weibull Type 2) as the level that impeded 50% (EC_{50}) of the females by egg rope production. A dose relationship of the fertility data also provided 74.5 µg/kg dw (95% CI, 40.1 and 108.9 µg/kg dw) as the EC_{50}-value that impeded 50% of the laid egg ropes from hatching (**Fig. 8**).

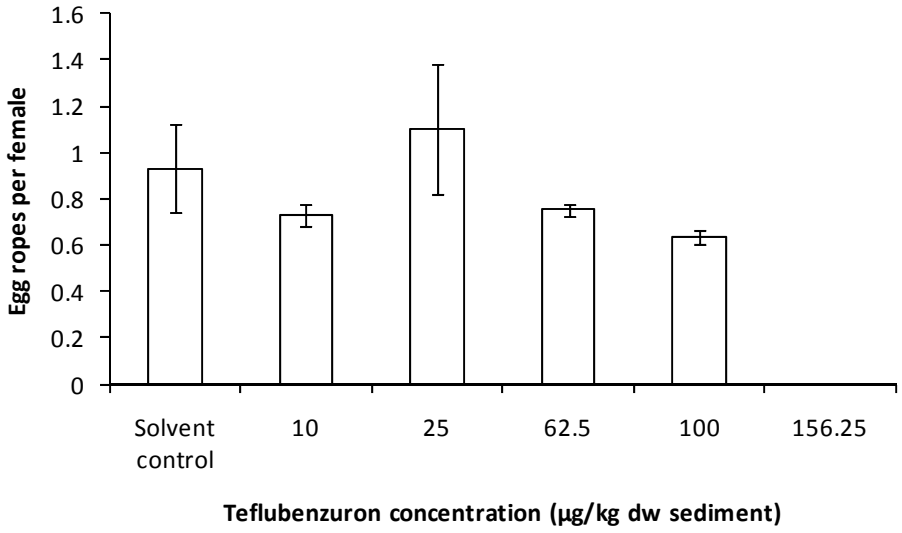

Fig.7 The number of egg ropes per female (±SD, n = 8) produced by the P generation of *C. riparius* exposed to different teflubenzuron concentrations in sediment. An EC_{50}-value of 112.7 µg/kg dw (95% CI, 82.05 and 143.4 µg/kg dw) was estimated (Weibull Type 2) [see also **Appendix I**].

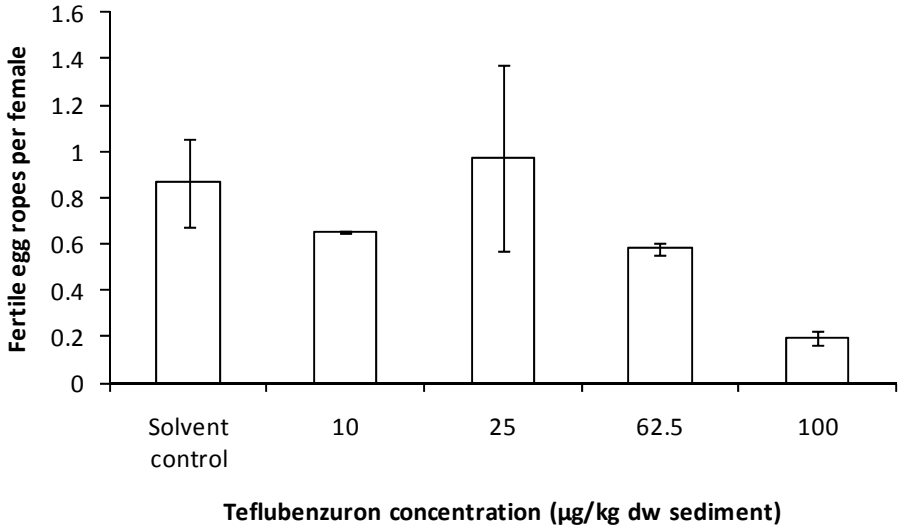

Fig. 8 Fertility (±SD, n = 8) of the P generation of *C. riparius* following static sediment spiked exposure with teflubenzuron showing a dose-response relationship with an EC_{50}-value of 74.5 µg/kg dw (95% CI: 40.1-108.9 µg/kg dw) [see also **Appendix I**].

Results of tebufenozide effects on the reproduction of *C. riparius* are presented in Fig. 9 indicating an EC_{50}-value of 23.8 µg/L (95% CI, 19.1 and 26.9 µg/L) for the fecundity and a

significant effect on the fertility at 26.2 µg/L. Additionaly, a dose-response analysis (Weibull Type 2) of the fertility data also yielded 23.0 µg/L (95% CI, 19.1 and 26.9 µg/L) as the EC_{50} concentration for this endpoint.

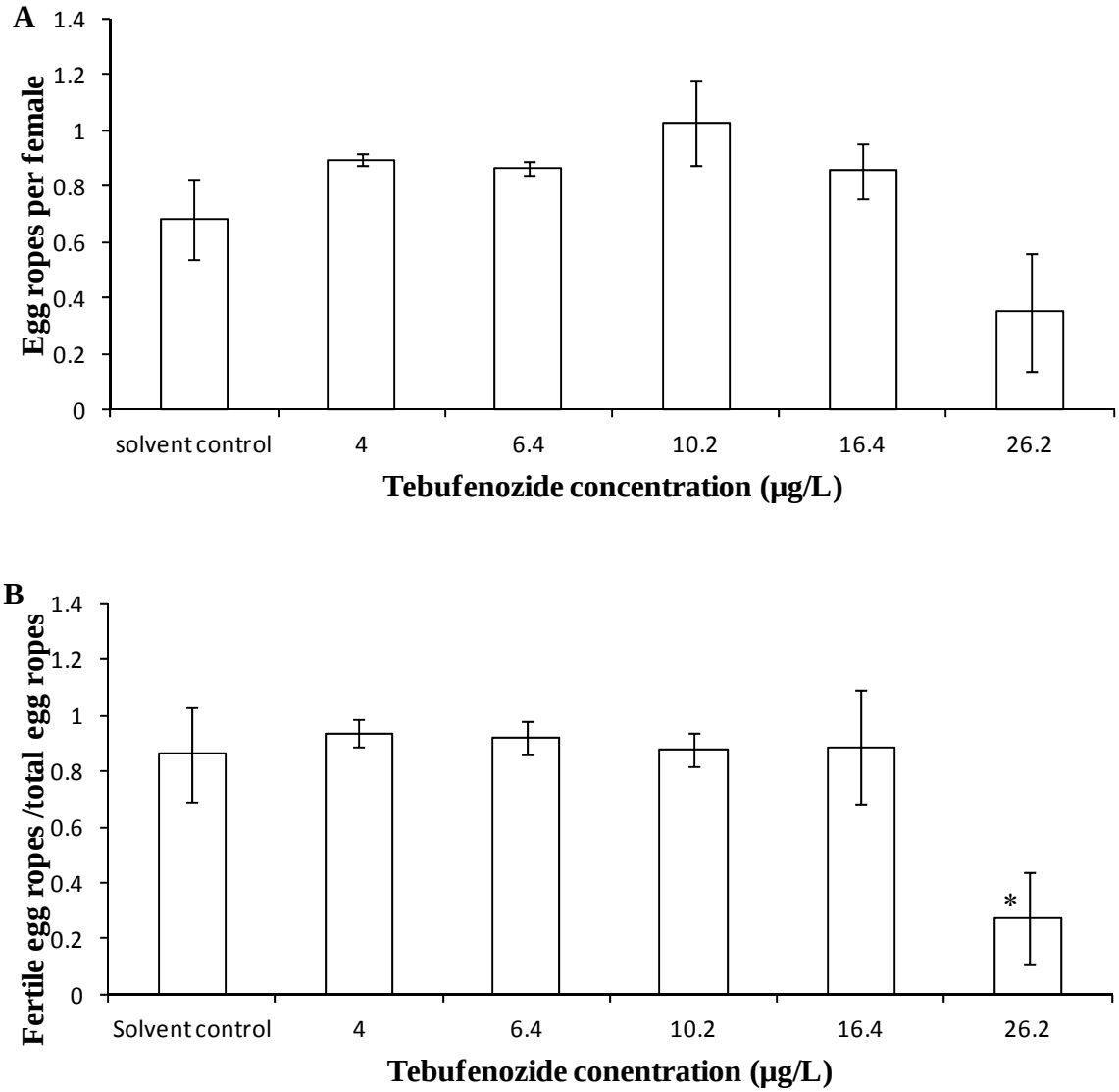

Fig. 9 Effects of sub-lethal tebufenozide concentrations on the reproduction of the P generation of *C. riparius* (A: fecundity; B: fertility) after a 28-day static exposure of first-instar larvae. An EC_{50}-value of 23.8 µg/L (95% CI, 19.1 and 26.9 µg/L) was estimated (Weibull Type 2) for the fecundity and asterisk denotes a significant difference compared to the solvent control (ANOVA, Dunnett's test p = 0.014) [see also **Appendix II**].

The effects of pyriproxyfen on reproduction were assessed at each of the selected temperature levels of 16 and 24°C. A significant decrease in the egg rope production was already present at 3 µg/L at each level compared to the corresponding solvent control (**Fig. 10**). EC_{50}-values of 3.1 µg/L (95% CI, 1.4 - 7.1) at 16°C and 2.7 µg/L (95% CI, 1.8 – 6.5) at 24°C were evaluated for this endpoint. The fertility in the solvent controls at each selected temperature was over 84%. In the exposed midges, the average fertility was above 69%. At 16°C and 10 µg/L pyriproxyfen, none of the egg ropes laid was fertile.

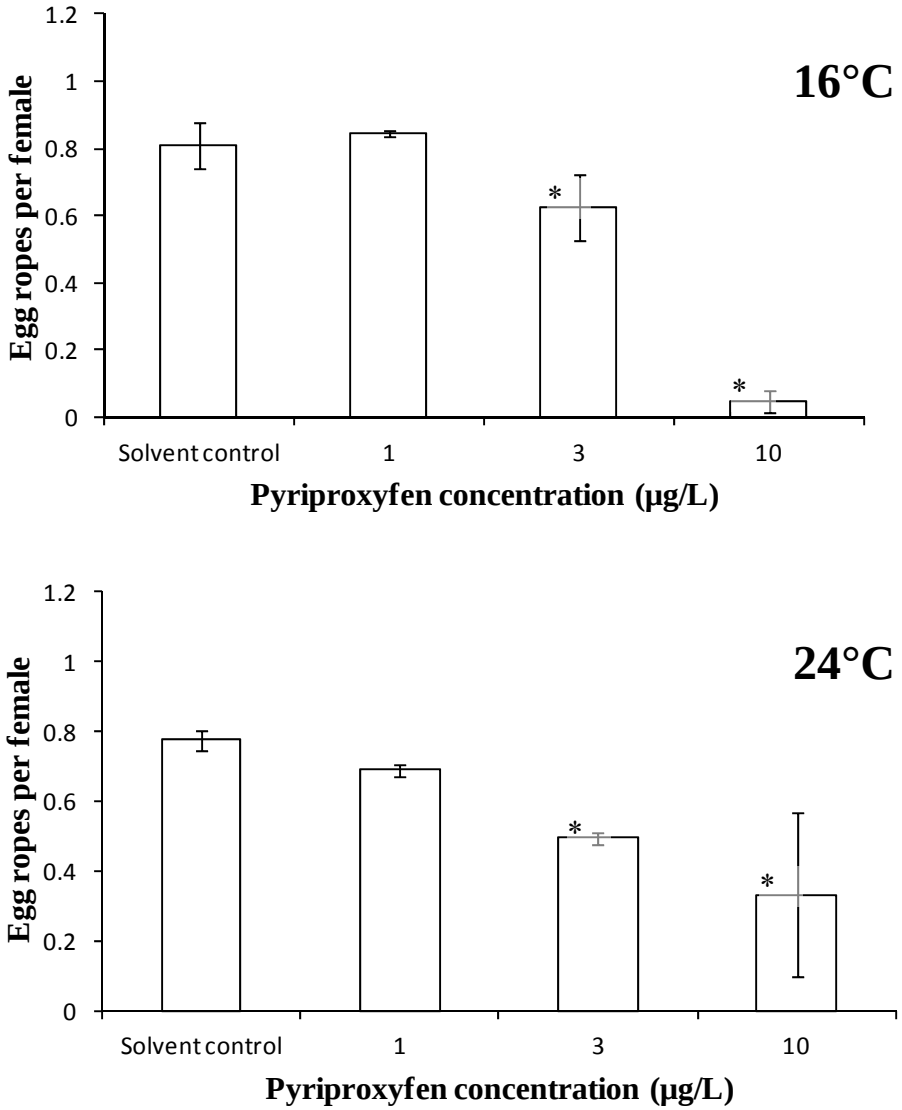

Fig. 10 Egg ropes per female of *C. riparius* in the P generation at 16 and 24°C after pyriproxyfen exposure under static conditions. Asterisks denote significant differences compared to the solvent control [see also **Appendix III**].

5 Discussion

5.1 Suitability of *C. riparius* to detect effects of potential EDCs in freshwater invertebrates

The studies presented in this thesis indicated that it is possible with *C. riparius*, a non-target freshwater insect, to assess potential effects of the selected IGRs on an aquatic macroinvertebrate species. *C. riparius* is a key, sexually reproductive species in the aquatic food chain and plays an important role in the freshwater ecosystem (detritus feeder and food source for many fish species and birds). Therefore, its suitability as a test species in addition to Crustacean assays (often used in ecotoxicity tests) is relevant for the risk characterization process. This, may have important implications in the ERA, especially for EDCs. This is also in agreement with the study of Taenzler et al. (2007), who proposed the same species as an aquatic invertebrate test species for EDC risk assessments. *C. riparius* was also proposed as a suitable organism in EDC-associated reviews with an emphasis on aquatic arthropods (OECD 2006). Hahn et al. (2001) also stated that the detection of sex-specific effects in *C. riparius* underlined its suitability as a model organism for investigating EDCs in aquatic insects. Furthermore, Soin and Smagghe (2007) reported that aquatic insects with flying adults would represent a long-term response through resistance evolution and population change; thus, these insects may react differently with continued exposure to a toxin or an EDC.

Although the choice of representative tests species was suggested by Breitholtz et al. (2006) as one of the 10 major issues for the improvement of ecotoxicological testing in ERA practices, until now many standard protocols use test species that are not the best representatives of the invertebrate community as a whole (Breitholz et al. 2006). Using chironomids (sexually reproducing organisms) as test organisms to assess endocrine-controlled sexual reproduction processes for EDCs would present an advantage, as reported in this work, when compared to studies using *Daphnia magna* (nonsexual reproductive organism). Daphnids predominantly reproduce via parthenogenesis and would therefore not be better suited for the assessment of endocrine control of sexual reproduction processes (Taenzler et al. 2007, Tassou and Schulz 2009). This problem was already pointed out by Barnthouse (1993), who stated that hazard identifications partly based on *Daphnia* tests may, for a range of chemicals, be of limited value for sexually reproducing species in the environment. For this reason, this thesis, which aimed to investigate the suitability of *C.*

riparius over two generations in assessing the risks of ED in aquatic insects, may be a useful amendment to improve ERA practices. This might be especially relevant if endocrine control of sexual reproduction processes in aquatic insects is considered.

5.2 Effects of the selected test substances

The aim of this thesis was to assess ED effects in a non-target freshwater insect through a full life-cycle test over two generations and hence acknowledge the suitability of *C. riparius* as a representative species of freshwater insects for EDC testing. Since there is currently no agreement on how to characterize and assess endocrine disruption (ED) in freshwater insects, the main hypothesis here was that long-term studies spanning all critical life stages of an organism over generations might, as also suggested by McKenney (2005), identify and characterize possible latent or cumulative effects occurring in an organism's life history. Therefore, these studies reported on the adverse effects of the different IGRs on *C. riparius* over two generations [**Appendices I, II, III]**. The results revealed significant impacts on the development and reproduction of *C. riparius*, two major processes that are controlled by the endocrine system, and suggest that the freshwater ecosystem structure and function may be affected once these compounds reach the system. The results of this thesis further contribute to answering the question asked by Taenzler et al. (2007): Are chironomids adequate test models to address the risk of potential endocrine effects of pesticides in freshwater invertebrates?

5. 2.1 Effects on the development of *C. riparius*

More specific information on the effects of each of the selected IGRs on the development of *C. riparius* has been reported in **Appendix I**, **II** and **III** and also published in peer- review journals. The results of the experiments summarized in this thesis indicate that the exposure over two generations (full life-cycle) to environmentally-relevant concentrations of the selected IGRs not only significantly affected the emergence ratio and the development rate of the midges but also showed the second (F1) generation of the midges to be more sensitive than the first (P) generation (**Fig. 3, 4, 5, 6**). This disruption of the developmental functions in *C. riparius* might suggest long-term ecological consequences (Tassou and Schulz 2011) considering the role that chironomids play in the freshwater ecosystem. Moreover, the

increased sensitivity of the second generation is in accordance with previous studies with *C. riparius* and some IGRs (Taenzler et al. 2007; Tassou and Schulz 2009) and emphasizes the importance of conducting tests at least over two generations. This should adequately predict the potential long-term impacts of substances that are persistent in the environment and which effects can be spread over generations of species (EDCs). This is also in accordance with the recommendation by Desneux et al. (2007) to consider sub-lethal effects of pesticides on arthropod physiology and behaviour for a complete analysis of pesticide impacts, in order to determine if the evaluation should be included in the registration process. The present thesis hence provided a hint to the fact that long-term tests of chronic toxicity with EDCs may yield additional information in order to improve upon the standard risk assessment. This standard is based on data from studies of acute or chronic exposure [**Appendix II**] that are often performed at environmentally unrealistic concentrations for a maximum one generation.

Although the protocol used here does not aim at explaining mechanistic processes caused by the selected IGRs, the reduction of the emergence ratio and the development rate of the midges might be a direct consequence of the mode of action of these compounds since development in chironomids is known to be under hormonal control. Furthermore, the data obtained with the ecdysone agonist tebufenozide on male development rate (**Fig. 5**) and male fraction (**Fig. 4**) over both generations revealed a trend to sex-specific effects that have already been observed with EDCs. Hahn et al. (2001) reported a sex-specific effect of tebufenozide in *C. riparius* after a semi-static exposure at 100 µg/L tebufenozide. This concentration is, however, four times higher than the highest concentration of 26.2 µg/L tebufenozide used in this study and the observed effect could be a consequence of the exposure scenario (semi-static vs static) or the exposure duration which is longer here (two generations) than the 8-day test duration reported by Hahn et al. (2001). The observation in this thesis is hence in accordance with observations by Reynolds et al. (2004) who reported a significant impairment of subsequent male reproduction function in the Lepidopterean *Spodoptera litura* with the effects manifested at lower ppm levels (0.5-2 ppm) of tebufenozide after larval exposure to sub-lethal doses.

The assessment performed at different temperature regimes [**Appendix III**] also revealed significant effects on the emergence ratio and development rate with the second generation showing greater sensitivity than the first generation (**Fig. 3 & 6**). This suggests that the present study may contribute to future investigations of possible interactions to be expected at

different temperature levels when IGRs act during the full life-cycle over two generations on biological processes in freshwater insects. This suggestion has been confirmed by the data showing for the first time a significant interaction effect between temperature, pesticide and generation ($p < 0.001$; **Table 1**) in a full life-cycle test (Tassou and Schulz 2012). This finding is very important since Holmstrup et al. (2010) remarked that although a large number of studies reporting temperature stress effects on aquatic organisms exist, studies on interactions of toxic compounds and temperature are scarce. This thesis provides the evidence that this test protocol could be suitable for the hazard identification and characterization with climate change-related variables and may contribute to filling in gaps of knowledge on the interactions between chemicals and temperature that hampers the extrapolation of laboratory toxicity data to ecosystems (Heugens et al. 2003). The interactions observed over two generations (**Table 1**) are of great importance for predicting the viability and distribution of populations in the field and may result in profound impacts on the whole ecosystems, considering the role those ectotherms organisms play in the aquatic food chains. This is in accordance with the suggestion by Vinebrooke et al. (2004) to investigate the interactive effects of multiple stressors (such as pollutants and temperature) and their mechanisms for predicting the tolerance limits, survival and productivity of ecthoterm populations and for modelling the effects of global climate change on aquatic organisms. However, this work also revealed that the variation of the temperature regime may per se be a source of additional stress for *C. riparius* (**Table 3; Fig 6**) indicating that a possible adaptation to the new temperature may also cost for ectotherms organisms.

Moreover, whether the exposure scenario was water spiking [**Appendices II & III**] or sediment spiking [**Appendix I**], the disruption of the developmental parameters was observed and revealed that *C. riparius* has an advantage over test species limited to a single media or matrix (e.g *Daphnia magna*) for assessing the potential adverse effects of EDCs. Biological impacts of these compounds on aquatic organisms can result through exposure via the water phase and/or the sediment (Taenzler et al. 2007; Tassou and Schulz 2011).

5.2.2 Effects on the reproduction of *C. riparius*

The assessment on the reproduction was determined by the number of egg ropes produced by the P generation and the fertility of these egg ropes (OECD 233). The results revealed that sub-lethal, environmentally-relevant concentrations of the selected IGRs significantly impair the fecundity and fertility of *C. riparius* (**Fig. 8, 9, 10 and Appendices I; II; III**). This

emphasizes the importance that must be given to reproduction as an additional endpoint in assessing EDCs. This is in accordance with the study by Desneux et al. (2007) who suggested adding sub-lethal effects on aquatic arthropods' biology and physiology to the direct mortality induced by pesticides for a complete analysis of their impacts. This is important considering the role that reproduction plays as the main process linking the individual to the population [**Appendix II**] and may also explain the interest which has been accorded to the assessment on reproduction in the adopted OECD guideline 233 in comparison to the standard chronic *Chironomus* study (OECD 2004).

Therefore, the data presented in this thesis may suggest biological implications on *C. riparius* for the ability to regulate its population (Tassou and Schulz 2011) once the IGRs reached the freshwater non-target ecosystem. This suggestion is in accordance with results reporting the reduction in oviposition of some IGR (CSI) treated females of different insect orders (Wilson and Cryan 1997; Valle et al. 2009) and the study by Desneux et al. (2007) who suggested that IGRs may induce more long-term effects on fecundity, than for example, neurotoxins. In addition, Gelbic and Rozsypalová (2012) reported that the toxicity of some IGRs (non-steroidal ecdysone agonists such as tebufenozide) could be used as alternative ways of regulating aquatic insect populations in developing countries. Population reduction in the field as a consequence of the application of IGRs has already been observed by Cadogan et al. (2002) who reported on the multiple year carry-over effects of tebufenozide.

The investigations on the reproduction at different temperature levels also revealed a dose-related decrease in the fecundity (**Fig. 10**) and indicated a factor of ≥ 6 between EC_{50}-values [**Appendix III; Tassou and Schulz 2012**] if the results in this thesis (conducted at 16 and 24°C) are compared to those obtained at the optimum temperature of 20°C in a previous study (Tassou and Schulz 2009). This is in accordance with the study by Oetken et al. (2009), who observed a significant decrease in the number of egg ropes by females of *C. riparius* between 20 and 23°C tributyltin treatments. This suggests an interaction effect between temperature and IGRs on the fecundity at temperature levels different from the usal optimum value of 20°C in the laboratory. It also confirms the suggestion by Jacobson et al. (2008) that elevated temperature may interact synergically with the fungicide fenarimol (for example, reduced the fecundity of the amphipod *Monoporeia affinis)*. Therefore, the results presented here revealed possible interaction effects between IGRs and temperature, which may impair the biological functions of aquatic insects and finally lead to severe consequences for the whole freshwater

ecosystem. Hence, the investigation on possible interaction effects between temperature and pesticides during a full-life cycle must be highly relevant particularly for the assessment of EDCs that mimic functions of endogenous hormones. This may be helpful in the context of global climate change-related variables: knowing how laboratory populations may behave with combined temperature and IGR effects during a full-life cycle might help to predict impacts that will happen in the field (Tassou and Schulz 2012**)**. This can also provide a new insight into the ERA of pesticides, particularly those interfering with the endocrine system of freshwater organisms that are ectotherms.

5. 2.3 Comparative effects of the IGRs on the life traits of *C. riparius*

The effects on developmental and reproductive traits of *C. riparius* observed during this thesis are summarized in **Table 5.** It gives an overview of the first study that documents the effects of different IGRs in a full life-cycle test over two generations. The results indicated that the selected IGRs, namely teflubenzuron (CSI), tebufenozide (non-steroidal ecdysone agonist) and pyriproxyfen (JHA), affected the endocrine function of *C. riparius* at sub-lethal concentrations but not in the same way (**Table 5**). While the JHA (pyriproxyfen) and the CSI (teflubenzuron) showed significant effect on the overall emergence ratio and development rate of midges, no effect on this endpoint was observed with the non-steroidal ecdysone agonist tebufenozide. Significant effects on the male development rate (**Fig. 5; Appendix II**) and on the male fraction in a treatment in the F1 generation (**Fig. 4; Appendix II**) were observed with tebufenozide but not for teflubenzuron and pyriproxyfen. Pyriproxyfen was also the only substance that produced significant effects on both fecundity and fertility of *C. riparius* when treatments were compared with control (ANOVA; Dunnett's test or William test), while the detection of effects of teflubenzuron and tebufenozide needed more robust statistical method (dose-response analysis by regression). Significant effects of pyriproxyfen were observed for each of the endpoints investigated (**Table 5**). In view of this analysis, perhaps the JHA pyriproxyfen might be the more problematic substance followed by the non-steroidal ecdysone agonist tebufenozide and the CSI teflubenzuron. This suggestion could on the one hand be a consequence of the spiking scenario (water spiking by pyriproxyfen and tebufenozide exposure vs. sediment spiking by teflubenzuron exposure) or, on the other hand, depend on the specific mode of action of each of the IGRs. This is in accordance with the study by Åkerblom et al. (2010) who reported that the exposure scenario (via water or sediment) is an important determinant for the behaviour and bioavailability of test

compounds. In addition, while effects of JHAs (e.g. Tatarazako et al. 2003; Dhadialla et al. 2005) and non-steroidal ecdysone agonists (Gelbic and Rozsypalová 2012) as consequences of endocrine disruption in freshwater arthropods were reported, effects of CSIs have been suggested to be non-endocrine (Oetken et al. 2004). This suggestion is, however, in contrast to other studies reporting endocrine effects of CSIs in different insect orders (Wilson and Cryan 1997; Hooper et al. 2005; Valle et al. 2009).

Table 5 Observed effects of selected IGRs on *C. riparius* during a full life-cycle test (two subsequent generations). As the tests with teflubenzuron and tebufenozide were conducted at 20°C, results for pyriproxyfen reported here are those published in Tassou and Schulz (2009), also performed at 20°C.

Endpoints	**Teflubenzuron (CSI)**	**Tebufenozide (ecdysone agonist)**	**Pyriproxyfen (JHA)**
P emergence rate	+	-	+
F1 emergence rate	+	-	+
P development rate (both sexes)	+	+a	+
F1 development rate (both sexes)	-	+a	+
P male fraction	-	-	-
F1 male fraction	-	+b	-
P fecundity*	-	-	+
P fertility*	-	+	+

+: significance; -: not significant a: significance only for males; b: significance only in one treatment; *: EC_{50}-values were estimated

5. 3 Relevance of multigenerational studies in chemical testing

The studies summarized in this thesis indicated that for all IGRs investigated, the second (F1) generation of *C. riparius* showed more sensitivity for the chosen functional endpoints than the first (P) generation. These findings showed for the first time, the suitability of multigenerational testing for various different groups of EDCs and strongly suggest a multigenerational study of at least two generations as an appropriate test method for better hazards identification and characterization in the environment. This suggestion is in accordance with a proposal by the OECD, which strongly encourages the need for at least two-generational exposure protocols (life-cycle toxicity testing) for aquatic arthropods in order to adequately predict the potential chronic effects of EDCs (OECD 2006).

Since multigenerational experiments involve all sensitive life stages of an organism, they provide, as shown here, additional and more population level-relevant sensitive data as a useful amendment for the risk assessment of chemicals with chronic and transgenerational effects such as EDCs. This is in accordance with the study by Raimondo et al. (2009) who suggested that the evaluation of population level and multigenerational effects is particularly important in the risk assessment of EDCs, because adverse effects may not be evident during the first generation of exposure. McKenney (2005) also suggested that longer-term studies spanning critical life stages over multiple generations can identify and characterize possible latent or cumulative adverse effects occurring in an organism's life history. The conduction of studies over several generations, as a standard practice to investigate effects of IGRs, was already suggested by Henrick et al. (1973). In addition, traditional risk assessment guidelines for agrochemicals still employ toxicity tests based on acute or chronic exposure for a maximum of one generation (OECD 2004a, b). This seems to be insufficient for detecting effects at the population level (Desneux et al. 2007; Kramer et al. 2011; Stark and Banks 2003) and, therefore, the present two-generational protocol may provide valuable information for a useful amendment to improve the current ERA, especially for EDCs. This two-generational protocol did not provide, in contrary to screening assays, information on the mode of action of a substance. However, as also suggested by Verslycke et al. (2007), from a risk assessment point of view, the distinction between endocrine or metabolic toxicity based on sound mechanistic evidence is not crucial and probably less important than identifying the most sensitive physiological endpoints that can lead to long-term impacts at population level with their ecological consequences.

6. Conclusion

Ecological risk assessment (ERA) of endocrine disruptors requires testing systems for the purposes of risk management that effectively help to identify and characterize suspected substances that might have adverse effects on populations. This objective seems, however, to be restricted by traditional risk assessment guidelines for agrochemicals that employ toxicity tests based on acute or chronic exposure for a maximum of one generation to detect effects relevant at the population level. Therefore, the full life-cycle test as described in this thesis might be relevant in the ERA of persistent chemicals and EDCs, the effects of which can be spread over generations. The test at different temperature levels revealed that this protocol may also help in predicting interaction effects between EDCs and temperatures that may occur in the field and hence may be a useful amendment for the risk assessment of EDCs even in the context of the climate change predictions.

However, the use of a test system and the choice of a representative test species including all sensitive life stages of an organism are only part of a complex set of requirements on rational ecotoxicological testing of EDCs. Some other aspects not investigated in this thesis, such as the investigation of genetic variation in populations or the use of mechanistic endpoints to understand the toxic mode of action, should undoubtedly be included in future investigations for the assessment of EDCs. For the statistical evaluation of the data, more robust test methods such as the regression analysis should sometimes be preferred to the hypothesis testing method (ANOVA, William or Dunnett's tests) that showed some limitations (e.g. for fecundity and fertility). These could help in the real implementation of the new European chemical legislation, REACH (Registration, Evaluation Authorization and Restriction of Chemicals).

7. References

Åkerblom N, Goedkoop W, Nilsson T, Kylin H. 2010. Particle-specific sorption/desorption properties determine test compound fate and bioavalability in toxicity tests with *Chironomus riparius*-High-resolution study with lindane. Environ. Toxicol. Chem. 29:1520-1528.

Albanis T, Allera A, Bachmann J, Barbaglio A, Berntsson P, Dittmann N, Carnevali DC, Ciceri F, Dagnac T, Duft M, Falandysz J, Galassi S, Hala D, Janer G, Jeannot R, Jobling S, King I, Klingmüller D, Kloas W, Kusk KO, Lavado R, Lo S, Lutz I, Oehlmann J, Oredsson S, Porte C, Rand-Weaver M, Sakkas V, Schmitt C, Schulte-Oehlmann U, Sugni M, Tyler C, van Aerle R, van Ballegoy C, Wollenberger L. 2006. COMPRENDO- Comparative research on endocrine disrupters- phylogenetic approach and common principles focussing on androgenic/anti-androgenic compounds. EU contract no. EVK1-CT-2002-00129. Final report. Pp: 1-58.

Allan IJ, Vrana B, Greenwood R, Mills GA, Roig B, Gonzalez C. 2006. A "toolbox" for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta 69:302-322.

Agency for Toxic Substances and Disease Registry. 2006. Methoprene- Toxicologic information about insecticides used for eradicating mosquitoes (West Nile Virus Control), April 2005. ATSDR, U.S. Departement of Health and Human Services, Washington, USA.

Baldwin WS, Bailey R, Long KE, Klaine S. 2001. Incomplete ecdysis is an indicato of ecdysteroid exposure in *Daphnia magna*. Environ. Toxicol. Chem. 20:1564-1569.

Barnthouse LW. 1993. Population-level effects. In: Ecological risk assessment. Lewis Publishers, Chelsea.

Bars R, Broeckaert F, Fegert I, Gross M, Hallmark N, Kedwards T, Lewis D, O'Hagan S, Panter GH, Weltje L, Weyers A, Wheeler JR, Galay-Burgos M. 2011. Science based guidance for the assessment of endocrine properties of chemicals. Regul. Toxicol. Pharm. 59:37-46.

Boudjelida H, Bouaziz A, Soin T, Smagghe G, Soltani N. 2005. Effects of ecdysone agonist halofenozide against *Culex pipiens*. Pest. Biochem. Physiol. 85:115-123.

Breitholtz M, Rudén C, Hansson SO, Bengtsson BE. 2006. Ten challenges for improved ecotoxicological testing in environmental risk assessment. Ecotoxicol. Environ. Saf. 63:324-335.

Cadogan BL, Scharbach RD, Krause RE, Knowles KR. 2002. Evaluation of tebufenozide carry-over and residual effects on spruce budworm (Lepidoptera: Tortricidae). J. Econ. Entomol. 95:578-586.

Clubbs RL, Brooks BW. 2007. *Daphnia magna* responses to a vertebrate estrogen receptor agonist and an antagonist: A multigenerational study. Ecotoxicol. Environ. Saf. 67:385-398.

deFur P, Crane M, Ingersoll C, Tattersfield L. 1999. Endocrine disruption in invertebrates: endocrinology, testing and assessment. SETAC Press, Pensacola.

deFur PL. 2004. Use of and role of invertebrate models in endocrine disruptor research and testing. ILAR J. 45:484-493.

Dhadialla TS, Retnakaran A, Smagghe S. 2005. Insect growth and development disrupting insecticides In: Press P. (ed/eds), comprehensive insect molecular science. Gilbert, L.I.; Kostas, I. Gill, S.S, 55-116.

Dhadialla TS, Carlson GS, LeDat P. 1998. New insecticides with ecdysteroidal and juvenile hormone activity. Annu. Rev. Entomol. 43: 545–569.

Desneux N, Decourtye A, Delpuech JM. 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52:81-106.

Ducrot V, Teixeira-Alves, Lopes C, Delignette-Muller ML, Charles S, Lagadic L. 2010. Development of partial life-cycle experiments to assess the effects of endocrine disruptors on the freshwater gastropod Lymnaea stagnalis: a case-study with vinclozolin. Ecotoxicology 19:1312-1321.

Duft M, Schulte-Oehlmann U, Tilmann M, Weltje L, Oehlmann J. 2005. Biological impact of organotin compounds on mollusks in marine and freshwater ecosystems. Coast. Mar. Sci. 29: 95-110.

European Commission (EC). 1996. European Workshop on the Impact of Endocrine Disruptors on Human Health and Wildlife. Proceedings from a Workshop, December2-4, 1996, Weybridge, UK Report reference EUR17549. Brussels, Belgium: European Commission.

European Commission (EC). 1999. Strategy for endocrine disrupting chemicals. See http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:51999DC0706:EN:HTML.

Falconer KE. 1998. Managing diffuse environmental contamination from agricultural pesticides: An economic perspective on issues and policy options, with particular reference to Europe. Agric. Ecosyst. Environ. 69:37-54.

Flynn K. 2011. Dietary exposure to endocrine-active pesticides: Conflicting opinions in a European workshop. Environ. Int. 37:980-990.

Gelbic I, Rozsypalová P. 2012. Autogeny induced by a nonsteroidal ecdysone agonist tebufenozide in the mosquito Culex quinquefasciatus. Physiological Entomol. 37:119-126.

Gourmelon A, Ahtiainen J. 2007. Developing test guidelines on invertebrate development and reproduction for the assessment of chemicals, including potential endocrine active substances-The OECD perspective. Ecotoxicology 16:161-167.

Hahn T, Liess M, Schulz R. 2001. Effects of the hormone mimetic insecticide tebufenozide on *Chironomus riparius* larvae in two different exposure setups. Ecotoxicol. Environ. Saf. 49: 171-178.

Hahn T, Schenk K, Schulz R. 2002. Environmental chemicals with known endocrine potential affect yolk protein content in the aquatic insect *Chironomus riparius*. Environ. Pollut. 120: 525-528.

Henrick CA, Staal GB, Siddall JB. 1973. Alkyl 3,7,11-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. J. Agric. Food Chem. 21: 354-359.

Heugens EHW, Jager T, Creyghton R, Kraak MHS, Hendriks AJ, van Straalen NM, Admiraal W. 2003. Temperature-dependent effects of cadmium on *Daphnia magna*: accumulation versus sensitivity. Environ. Sci. Technol. 37:2145-2151.

Holmstrup M, BindesbØl AM, Oostingh GJ, Duschl A, Scheil V, Köhler HR, et al. 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Sci. Total Environ. 408:3746-3762.

Hommen U, Baveco JMH, Galic N, van den Brink PJ. 2010. Potential application of ecological models in the European environmental risk assessment of chemicals I: Review of protection goals in EU directives and regulations. Integr. Environ. Assess. Manag. 6:325-337.

Hooper HL, Sibly RM, Hutchinson TH, Maund SJ. 2005. Joint effects of density and a growth inhibitor on the life history and population growth rate of the midge *Chironomus riparius*. Environ. Toxicol. Chem. 24:1140-1145.

Jacobson T, Prevodnik A, Sundelin B. 2008. Combined effects of temperature and a pesticide on the Baltic amphipod *Monoporeia affinis*. Aquat. Biol. 1:269-276.

Kramer VJ, Etterson MA, Hecker M, Murphy CA, Roesijadi G, Spade DJ, Spromberg JA, Wang M, Ankley GT. 2011. Adverse outcome pathways and ecological risk assessment: bridging to population-level effects. Environ. Toxicol. Chem. 30:64-76.

Kropp RK, Pinza MR, Blanton ML. 2004. Draft detailed review paper on aquatic arthropods in life cycle and two- generation toxicity tests. BATTELLE, November, 10 2004, Ohio.

Marty MS, Carney EW, Rowlands JC. 2011. Endocrine disruption: Historical perspectives and its impact on the furure of toxicology testing. Toxicol. Sci. 120:93-108.

McKenney LC. 2005. The influence of insect juvenile hormone agonists on metamorphosis and reproduction in estuarine crustaceans. Integr. Comp. Biol. 45: 97-105.

Migula PJ. 2005. Ecotoxicology, Invertebrate in: Philip Wexler (2.ed.), Encyclopedia of toxicology. Elsevier, Oxford, pp. 133-137.

Mu XY, Leblanc GA. 2002. Environmental anti-ecdysteroids alter embryo development in the crustacean *Daphnia magna*. J. Experim. Zool. 292:287-292.

Newman MC, Crane M, Holloway G. 2006. Does pesticides risk assessment in the European Union assess long-term effects? Rev. Environ. Contam. Toxicol. 187:1-65.

Oda S, Tatarazako N, Watanabe H, Morita M, Iguchi T. 2005. Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. Chemosphere 61:1168-1174.

Oehlmann J, Schulte-Oehlmann U. 2003. Endocrine disruption in invertebrates. Pure Appl. Chem. 75:2207-2218.

Oehlmann J, Schulte-Oehlmann, U, Bachmann J, Oetken M, Lutz I, Kloas W, Ternes TA. 2006. Bisphenol A induces superfeminization in the ramshorn snail *Marisa cornuarietis* (gastropoda: prosobranchia) at environmentally relevant concentrations. Environ. Health perspect. 114:127-133.

Oetken M, Bachmann J, Schulte-Oehlmann U, Oehlmann J. 2004. Evidence for endocrine disruption in invertebrates. Int. Rev. Cytol. 236:1–44.

Oetken M, Jagodzinski LS, Vogt C, Jochum A, Oehlmann J. 2009. Combined effects of chemical and temperature stress on *Chironomus riparius* populations with differing genetic variability. J. Environ. Sci. Health Pt A 44: 955-962.

Organisation for Economic Co-operation and Development. 2004a. Guideline for the testing of chemicals, sediment-water Chironomid toxicity test using spiked sediment, OECD 218, adopted 13 April 2004.

Organisation for Economic Co-operation and Development. 2004b. Guideline for the testing of chemicals, sediment-water Chironomid toxicity test using spiked water, OECD 219, adopted 13 April 2004.

Organisation for Economic Co-operation and Development. 2006. Detailed review paper on aquatic arthropods in life cycle toxicity tests with an emphasis on developmental,

reproductive and endocrine disruptive effects. OECD document, No 55, OECD, Paris, France.

Organisation for Economic Co-operation and Development. 2010. Guidelines for the testing of chemicals, sediment-water Chironomid life –cycle test using spiked water or spiked sediment. OECD 233, adopted 22 July 2010.

Pennak R. 1989. Fresh-water invertebrates of the United States. Wiley,New York.

Raimondo S, Hemmer BL, Goodman LR, Cripe GM. 2009. Multigenerational exposure of the estuarine Sheepshead minnow (Cyprinodon Variegatus) to 17β-estradiol. II. Population-level effects through two life Cycles. Environ. Toxicol. Chem. 28:2409-2415.

Rasmussen JB. 1984. The life-history, distribution, and production of *Chironomus riparius* and *Glyptotendipes paripes* in a prairie pond. Hydrobiologia 119:65-72.

Reynolds SE. 1987. The cuticle, growth and moulting in insects:the essential background to action of acylurea insecticides. Pest. Sci. 20:131-146.

Reynolds SE, Seth RK, Kaur JJ, Rao DK. 2004. Effects of larval exposure to sublethal concentrations of the ecdysone agonists RH-5849 and tebufenozide (RH-5992) on male reproductive physiology in *Spodoptera litura*. J. Insect Physiol. 50:505-517.

Ristola T, Parker D, Kukkonen JVK. 2001. Life cycle effects of sediment-associated 2,4,5-trichlorophenol on two groups of the midge *Chironomus riparius* with different exposure histories. Environ. Toxicol. Chem. 20:1772-1777.

Schulte-Oehlmann, U, Tillmann M, Markert B, Oehlmann J, Watermann B, Scherf S. 2000. Effects of endocrine disrupters on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: triphenyltin as a xeno-androgen. Ecotoxicology 9:399-412.

Smagghe G, Dhadialla TS, Lezzi M. 2002. Comparative toxicity and ecdysone receptor affinity of nonsteroidal ecdysone agonists and 20-hydroxyecdysone in *Chironomus tentans*. Insect Biochem. Molecular Biol. 32:187-192.

Soin T, Smagghe G. 2007. Endocrine disruption in aquatic insects: a review. Ecotoxicology. 16: 83-93.

Stark JD, Banks JE. 2003. Population-level effects of pesticides and other toxicants on arthropods. Annu. Rev. Entomol. 48:505-519.

Süß A, Bischoff G, Mueller CW, Buhr L. 2006. Chemical and biological monitoring of the load of plant protection products and of zoocenoses in ditches of the orchard region "Altes Land". Nachrichtenbl. Deut. Pflanzenschutzd 58:28-42.

Taenzler V, Bruns E, Dorgerloh M, Pfeifle V, Weltje L. 2007. Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. Ecotoxicology 16:221-230.

Tatarazako N, Oda S, Watanabe H, Morita M, Iguchi T. 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53:827-833.

Tassou KT, Schulz R. 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotoxicol. Environ. Saf. 72:1058-1062.

Tassou KT, Schulz R. 2011. Two-generation effects of the chitin synthesis inhibitor, teflubenzuron, on the aquatic midge *Chironomus riparius*. Ecotoxicol. Environ. Saf. 74:1203-1209.

Tassou KT, Schulz R. 2012. Combined effects of temperature and pyriproxyfen stress in a full life-cycle test with *Chironomus riparius* (Insecta). Environ. Toxicol. Chem. 31:2384-2390.

Taylor S, Blake N. 2009. Literature reviews on ecotoxicology of chemicals with a special focus on plant protection products: Available protocols for testing the effects of chemicals against aquatic invertebrates other than crustacean. Report for EFSA. Report number CEA.468. Final report, 01 September 2009. Pp: 1-93.

Valle D, Belinato TA, Martins AJ, Lima JB, de Lima-Camara TN, Peixoto AA. 2009. Effect of the chitin synthesis inhibitor triflumuron on the development, viability and reproduction of *Aedes aegypti*. Mem. Inst. Oswaldo Cruz, Rio de Janeiro 104:43-47.

Verslycke TA, Ghekiere A, Raimondo S, Janssen C. 2007. Mysid crustaceans as standard models for the screening and testing of endocrine-disrupting chemicals. Ecotoxicology 16: 205-219.

Vinebrooke RD, Cottingham KL, Norberg J, Scheffer M, Dodson SI, Maberly C, Sommer U. 2004. Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. Oikos 104: 451-457.

Wang HY, Olmstead AW, Li H, LeBlanc GA. 2005. The screening of chemicals for juvenoid-related endocrine activity using the water flea Daphnia magna. Aquat. Toxicol., 74:193-204.

Watts MM, Pascoe D, Carroll K. 2001. Chronic exposure to 17 alpha-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diptera: Chironomidae). Aquat. Toxicol. 55:13-24.

Wilson JD, Morris AJ, Arroyo BE, Clark SC, Bradbury RB. 1999. A review of the abundance and diversity of invertebrate and plant foods of granivorous birds in northern Europe in relation to agricultural change. Agricul. Ecosyst. Environ. 75:13-30.

Wilson TG, Cryan JR. 1997. Lufenuron, a chitin-synthesis inhibitor, interrupts development of *Drosophila melanogaster*. J. Exp. Zool. 278:37-44.

Appendix I

Two-generation effects of the chitin synthesis inhibitor, teflubenzuron on the aquatic midge *Chironomus riparius*

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Abstract

Teflubenzuron, a chitin synthesis inhibitor was used in a two-generation test with *Chironomus riparius* (Insecta) to assess effects over a full life cycle from the first-instar larvae in the parental (P) generation until emergence in the subsequent F1 generation. Sediment spiked with teflubenzuron ranging from 10 µg/kg to 390.6 µg/kg sediment dry weight (dw) was used. EC_{50}-values for fecundity and fertility were 112.7 and 74.5 µg/kg dw, respectively. Significant adverse effects were observed compared to the solvent control for emergence rate ($P < 0.01$) and also for developmental rate. No observed effect concentrations values were lower for emergence rate in the F1 generation (62.5 µg/kg dw) than in the P generation (100 µg/kg dw), demonstrating that the F1 generation was more affected than the P generation.

Thus, this two-generation test may help detecting population level effects as an amendment to the risk assessment for chronic exposures to endocrine disrupting compounds.

Keywords: Teflubenzuron; CSI; *Chironomus riparius*; two-generation test; population-level effects.

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Formal assurance

The authors assure that no studies involving humans or experimental animals were conducted without accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

Introduction

Insect Growth Regulators (IGR) represent a group of insecticides, often referred to as third generation insecticides, which were developed to intentionally mimic, block or otherwise interact with the hormonal system of insects (Oetken et al. 2004). These include the juvenile hormone analogues, the ecdysone agonists and the chitin synthesis inhibitors (CSIs) (Graf 1993; Tunaz and Uygun, 2004).

CSIs are benzoylphenylurea (BPUs) compounds that interfere with larval development, disturbing moulting and resulting in deformations in the cuticle (Reynolds, 1987; Graf, 1993). These adverse effects are due to CSIs interference with chitin synthesis, diminishing the amount of this polymer in the insect cuticle (Ishaaya and Casida, 1974; Post et al. 1974). Adults derived from CSI-exposed larvae can present a series of sub-lethal physiological abnormalities that might ultimately diminish physical and reproductive capacities (Mondal and Parween, 2000; Desneux et al. 2007). Effects of CSIs vary according to species, the developmental stage at the time of application, the kind of compound and the administered dose (Wilson and Cryan, 1997; Mulla et al. 2003). Several studies in the literature reported about the direct effects of CSIs on insect pest organisms, such as mortality or adult emergence inhibition (Rehini and Soltani, 1999; Su et al. 2003; Batra et al. 2005), but the long-term effects of those compounds on the surviving adults or the next generation of non-target organisms with their ecological implications at the population level remain less considered (Desneux et al. 2007). The only exceptions are studies that reported effects of BPUs (e.g. diflubenzuron; lufenuron) that demonstrated their latency of effects on aquatic insect populations in freshwater mesocosms (Fairchild et al. 1996; Brock et al. 2009), however, these studies did not include transgenerational effects.

Traditional risk assessment guidelines for agrochemicals employ toxicity tests based on acute or chronic exposure for a maximum of one generation (OECD, 2004), which seem to be insufficient for detecting effects at the population level (Desneux et al. 2007; Stark and Banks, 2003). In particular for freshwater insects, new suitable test systems for IGRs have to be developed or currently used protocols adapted (OECD, 2006). Multigenerational reproduction assessment seems to be an approach enabling the detection and characterization of endocrine disrupting chemicals (OECD, 2006). Henrick et al. (1973) already suggested to perform studies over several generations as a standard practice to investigate effects of IGRs. The present paper employs a test design with *Chironomus riparius* over two generations

(approximately 56 days), in order to assess adverse effects over a full life cycle from the first-instar larvae of the parental (P) generation until emergence in the subsequent F1 generation (Taenzler et al. 2007; Tassou and Schulz, 2009). *C. riparius*, a sediment dwelling freshwater organism, whose first life stages take place under aquatic conditions, can be used for water or sediment exposure, which is an advantage over tests limited to a single media or matrix since biological impacts on aquatic organisms can result through exposure via the water phase and/or the sediment.

The test protocol is an enhancement of the existing guideline for testing of chemicals, the sediment-water chironomid toxicity test using spiked sediment (OECD, 2004). The extension includes in addition to the extended exposure over an entire life cycle, new endpoints such as fecundity and fertility for the P generation and emergence ratio and development rate for the F1 generation. Teflubenzuron, a CSI used as a pesticide on crops and as a biocide by fish farmers was selected as the test compound for this study. In agriculture, teflubenzuron is used for management of pest insects on various crops, such as fruit orchards, vine, cotton and vegetables. Teflubenzuron had been detected in shallow groundwater below cotton fields in Brazil at levels up to 2.6 µg/L (Carbo et al. 2008). According to the Scottish Environmental Protection Agency, around 90% of the ingested teflubenzuron in fish farm is evacuated from fish via faeces in the period immediately following treatment, with the remaining part entering the environment in the form of uneaten waste feed (SEPA, 1999). Thus, the purpose of this study was to determine the effects of teflubenzuron on the sediment dwelling organism, *C. riparius*, a non-target aquatic insect under laboratory conditions. CSIs have not previously been tested using a two-generation test to check for accumulation or carry-over effects on development or reproduction of aquatic organisms, however, long-term observations performed in mesocosm tests with compounds like diflubenzuron and lufenuron do exist (Sundaram et al. 1991; Fairchild et al. 1996; Brock et al. 2009). Delayed responses in emergence and abundance of aquatic macroinvertebrate arthropods community as treatment-related effects were observed two to six weeks after lufenuron application (Brock et al. 2009). In outdoor aquatic ecosystems, Sundaram et al. (1991) observed reduced abundance of mayfly and dragonfly nymphs approximately 21 to 34 days after application of diflubenzuron.

Materials and methods

Test species

The test organism used in this full life cycle test over two generations is *C. riparius*. It belongs to the family Chironomidae (true or non-biting midges) that belongs to the order Diptera and consists of several thousand species with a worldwide distribution (Pennak, 1989). Chironomids are called ‘‘deposit’’ or ‘‘ingestion-feeders’’ because they ingest particles from the sediment and the detritus as well as bacteria and algae (Rasmussen, 1984). They undergo a complete metamorphosis consisting of the egg stage, four larval stages, pupa and imago. The larvae are aquatic, adult midges emerge within 15–17 days after egg hatching (Watt et al. 2001) and live for about 4 days (Ristola et al. 2001). Under breeding conditions in the laboratory, males emerged 24–48 h earlier than females; a phenomenon which is known as protandry (Armitage et al. 1995). The adult's sex can be easily determined by their body structure. Sexual reproduction in *C. riparius* provides advantage compared to the organisms often used in assessing impacts upon endocrine controlled reproductive processes such as, *Daphnia magna* with a parthenogenetic reproduction. Moreover, the large amount of ecotoxicological data for chironomids can lend support to comparative studies.

Test item

Teflubenzuron (TFB) (1-(3,5-dichloro-2,4-difluorophenyl)-3-(2,6-difluorobenzoyl)-urea) is an insecticide from the fungal-derived group of acylureas also known as benzoylphenylureas (BPUs). Like other BPUs, TFB act on larval stages of exposed organisms, which usually become unable to survive the next moult. Ovicidal activity, achieved through the inhibition of chitin deposition in the developing larvae, also accounts for the efficacy of these compounds (Graf, 1993). TFB is a chitinase inhibitor insecticide, interfering with the deposition of chitin in the arthropod cuticle. Chitin is an essential constituent of the insect exoskeleton, the cuticle, which is an extracellular matrix covering the animal. It is produced by the glycosyltransferase chitin synthase that probably together with co-factors forms macromolecular structure known as the plasma membrane plaques (Mussian et al. 2009). Several mesocosm studies with BPUs like diflubenzuron and lufenuron have demonstrated that both representatives of crustaceans (particularly copepods and macrocrustaceans) and insects belong to the sensitive taxonomic groups because of their exoskeletons (Fairchild 1996; Brock et al. 2009). TFB has a moderate octanol: water partition coefficient of 2×10^4 at pH 7 and a relatively low water solubility

(0.01 mg/L at 20°C) which means that it tends to remain largely bound to sediment and organic material in the environment (Baird, 1998). The results from long-term site monitoring in Scotland to determine the fate and dispersion of teflubenzuron under normal treatment conditions using a low dispersion have reported a half-life of 104 to 123 days (Trouw, 1999), based on which SEPA (1999) has established a half-life of 115 days. The environmental quality standards (EQS) of TFB in sediments have been defined by SEPA (1999) as 2 µg/kg dry weight (average over the top 5 cm of a core applied as a maximum allowable concentration (MAC) to surface sediment outside the allowable effects area) and 10 µg/g dry weight (average of the top 5 cm of a core applied as an average value within the allowable zone of effects).

General test method

The methods used here largely follow those described in Tassou and Schulz (2009) with the difference that the application scenario was via sediment according to OECD 218 (2004). After preparation of the test vessels, the sediment-water system was left under gentle aeration (two bubbles per second) for two days prior to addition of 20 first-instar larvae of the P and F1 generation, respectively. During the addition of the first instars larvae to test vessels, and the following 24 h, aeration was stopped in order to allow the larvae to settle within the sediment. The first-instar larvae were exposed to six nominal test concentrations (10, 25, 62.5, 100, 156.3, and 390.6 µg/kg) of TFB including one solvent control made with acetone. Eight replicates were used for each treatment, as well as solvent control under static condition for one generation time of C. *riparius*. A stock solution of TFB has been prepared by dissolving the active ingredient in acetone for each concentration. Aliquots (10 mL) of stock solution or acetone (for solvent control) have been mixed thoroughly with 100 g of fine quartz sand for each concentration. The solvent has then been allowed to evaporate under fume hood for 1.5 h to remove it totally from the sand, which has then been mixed with the suitable amount of artificial sediment and water per test concentration to assure homogeneity. The overlying water (400 mL M4-medium) was then added with care so as not to disturb the sediment. The spiking procedure of the sediment and the incubation conditions were the same for both P and F1 generation prior to the addition of the first-instar larvae. The nominal test concentrations were calculated on the basis of dry weight of sediment and prepared by the dilution of a stock solution made in acetone with acetone. The larvae were fed on a daily basis with commercial fish-food (Tetra Min®). During the first 10 days, each vessel received 0.2 mL of a suspension

(equivalent to 0.5 mg fish-food per larvae per day) made with 6 g finely ground food and 120 mL M4-Medium. For the rest of the experiment, 0.4 mL suspension was added on a daily basis per test vessel. Once 50% of the midges emerged or food was observed on the sediment's surface, the food ratio was reduced.

The development time, the total number of fully emerged midges, and their sexes were determined for the P generation. The criterion for validity was set at a minimum of 70% of emerged adult midges within the solvent control (OECD, 2004). The adult midges were transferred with an exhauster into breeding cages (50 cm x 50 cm x 50 cm) where they can swarm, mate and oviposit into 2-L glass crystallization dish filled with 1 L non-aerated M4-medium and 300 g moist spiked artificial standard non-sterilized sediment. The egg ropes were collected daily from the crystallization dishes and kept in 12-well microtiter plates filled with water from the respective dish for assessing of fecundity and fertility. To obtain the F1 generation, egg ropes from one or two consecutive days around days 19 of the first generation phase were collected. After hatching, 20 first-instar larvae were inserted into each of the newly prepared test vessels for the F1 generation phase. Both the P and F1 generation were each investigated for a 28–day test period. Besides the endpoints already defined by the OECD guidelines 218 (OECD, 2004), the present study includes endpoints such as fecundity and fertility of the P generation and emergence ratio and development rate of the F1 generation.

Exposure to both generations was performed in a laboratory at 20 ± 2 °C with a 16:8 h light/dark regime and a light intensity of 708 ± 50 lx. An one hour dusk phase was generated using 15 W lamps for the mating or copulation of the adult midges in the breeding cages and humidity was maintained at 70 ± 10% relative air humidity.

Chemical analysis

The overlying water and pore-water were taken from additional test vessels at the start and the end of the test for both generations. Centrifugation at 10000 g and 4 °C for 30 min was done to extract pore-water from the sediment. For the analysis of sediment samples, the entire content of each vessel has been extracted twice with acetone using 1 mL solvent for 1 mg of sediment. The extraction procedure took place by adding the respective amount of acetone to the sediment and shaking the mixture for about 30 min before filtration. Overlying water, pore-water and filtrate from extraction were then combined into one sample and immediately

frozen at -20°C until analysis in water by a high-performance hybrid triple quadrupole/linear ion trap mass spectrometer (LC/MS/MS Mass Spectrometer 4000 QTRAP®) equipped with an Agilent 1100G1329A autosampler (AB Sciex Instruments), and an Agilent 1100 G1311A pump. MeOH LC/MS Grade (solvent A) and water purified with a MiliQ system (Millipore, Schwalbach, Germany) (solvent B) were used as eluents. Subsequently, 20 µL of the aqueous sample were injected into the system equipped with Merck Chromolith Performance column (100-4.6 mm RP 18e) after filtration through a 0.2 µm Nylonfilter. The Agilent 1100 LC Quaternary Pump was set at a flow rate 700 µl/min. The gradient started with 30% A and 70% B for 1 minute, was raised to 85% A in 5 minutes and hold for 8 min and then raised to 100% A in 6 min. The analytes were ionized using a Turbo Spray. The ions were detected in the scan range of 2200 in the negative mode. Stock solutions have also been measured and these measurements confirmed the applied amount of substance during the spiking process. The results are summarized in Table1.

Table 1: Nominal and measured concentrations of Teflubenzuron measured in stock solutions (S) and in water after sediment spiking.

Sample	Nominal concentration	Measured concenrtation (day 0)	Measured concentration (day 28)
S1	27.9 ng/mL	31.7 ng/mL	-
S2	27.9 ng/mL	25.8 ng/mL	-
S6	71.4 ng/mL	72.3 ng/mL	-
Solvent control	< LOD	< LOD	< LOD
10 µg/kg dw	10 µg/kg dw	0.028 ng/mL	< LOD
390. 6 µg/kg dw	390.6 µg/kg dw	7.36 ng/mL	9.81 ng/mL

LOQ: limit of quantification (LOQ = 0.15 ng/mL); LOD: limit of detection (LOD = 0.04 ng/mL); dw: dry weight of sediment

Data analysis

Values of the emergence rate for both P and F1 generations were arcsin-sqrt transformed to match normal distribution and to equalise variances before conducting an ANOVA. Dunnett's posthoc test was used to compare the treatment groups with the solvent control. A chi-square test was used to compare the percentage of larvae which did not emerge from teflubenzuron-treatments and the control. For the sex ratio, Fisher's exact test (with Bonferroni adjustment method) was performed to identify any statistical significance to the solvent control. For the fecundity and fertility, a dose-response relationship was investigated with the log-logistic regression model (Weibull Type 2).

Results

The effect thresholds for all endpoints evaluated in the P and F1 generation have been summarised in Table 2.

Table 2: NOEC and LOEC values for TFB generated by the full life cycle test over two generations with *C. riparius* (P: parental generation, F1: filial generation).

Endpoints	NOEC values [µg/kg dw]	LOEC values [µg/kg dw]
P emergence rate	100	156.3
P development rate	62.5	100
P sex ratio	ns	ns
P fecundity	ns	ns
P fertility	ns	ns
F1 emergence rate	62.5	100
F1 development rate	ns	ns
F1 sex ratio	ns	ns

ns: not significant

Emergence ratio

A dose–related decrease of the emergence of *C. riparius* (Fig. 1A) was observed in the P generation at the test concentrations ≥ 100 µg/kg dry weight of sediment (dw) compared to the solvent control. About 83% of the inserted larvae emerged in the solvent control at the end of the exposition time to teflubenzuron. At 156.3 µg/kg dw LOEC (Table 2), the emergence rate of inserted midges was adversely affected compared to the solvent control (Fig. 1A). For the F1 generation 91% of the inserted larvae in the solvent control emerged and a significant decrease of the emergence rate has been observed at the test substance concentration of 100 µg/kg dw (Fig. 1B). The NOEC for the F1 generation (62.5 µg/kg dw) was therefore lower than in the P generation (Table 2) indicating that the F1 generation showed stronger effects.

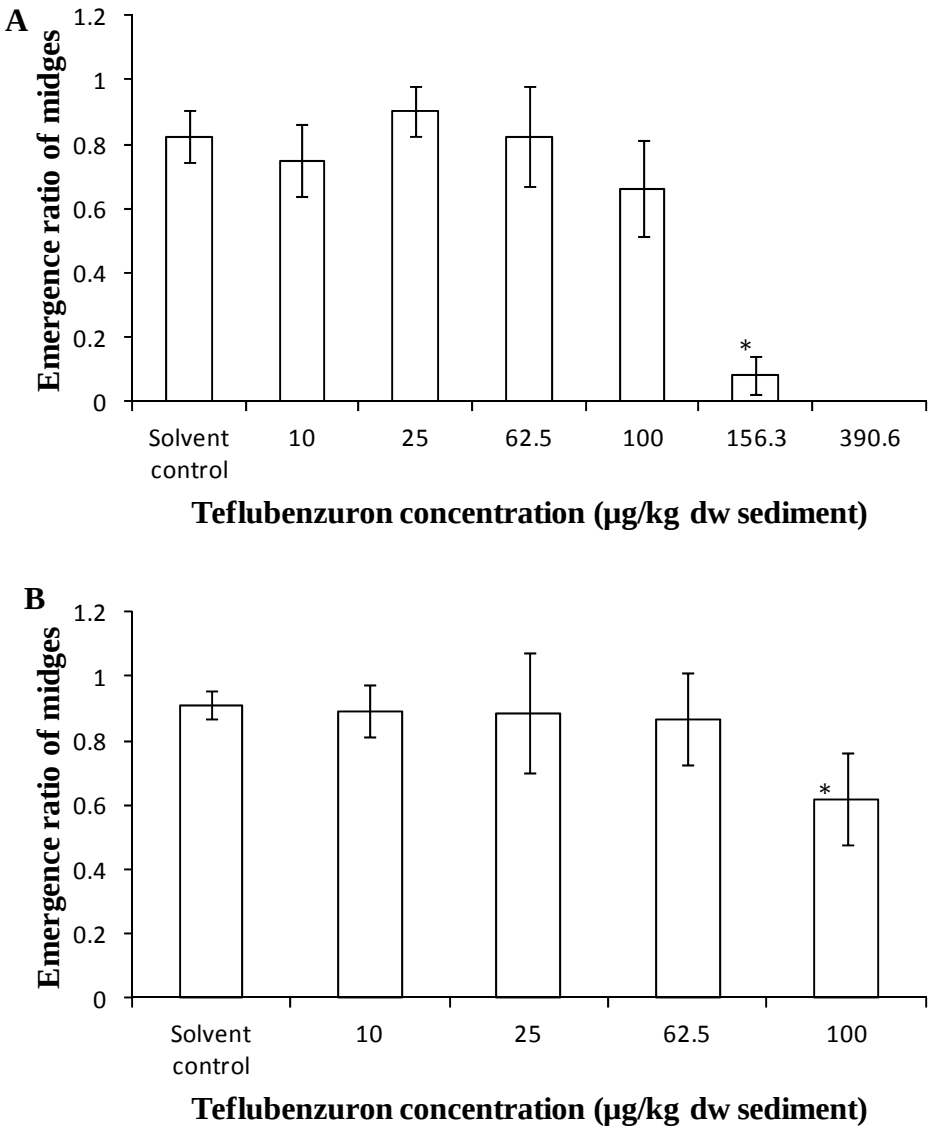

Fig. 1 Emergence rate (±SD, n = 8) of the P generation (A) and the F1 generation (B) of a *C. riparius* test using sediment spiked with teflubenzuron. Asterisks denote a significant difference compared to the solvent control (ANOVA+Dunnett's test $p < 0.001$).

Mortality of inserted larvae

In the P generation, a dose-related mortality of inserted first-instar larvae was observed in treatments ≥ 100 µg/kg dw. At the highest test concentration of 390.6 µg/kg dw, none of the inserted larvae emerged until the end of the exposure time (Fig. 2A). In the subsequent F1 generation, mortality was significantly higher in the 100 µg/kg dw treatment compared to the

solvent control (Fig. 2B). Mortality increased slightly from 32% to 38 % at 100 µg/kg dw when comparing the P generation to the F1 generation.

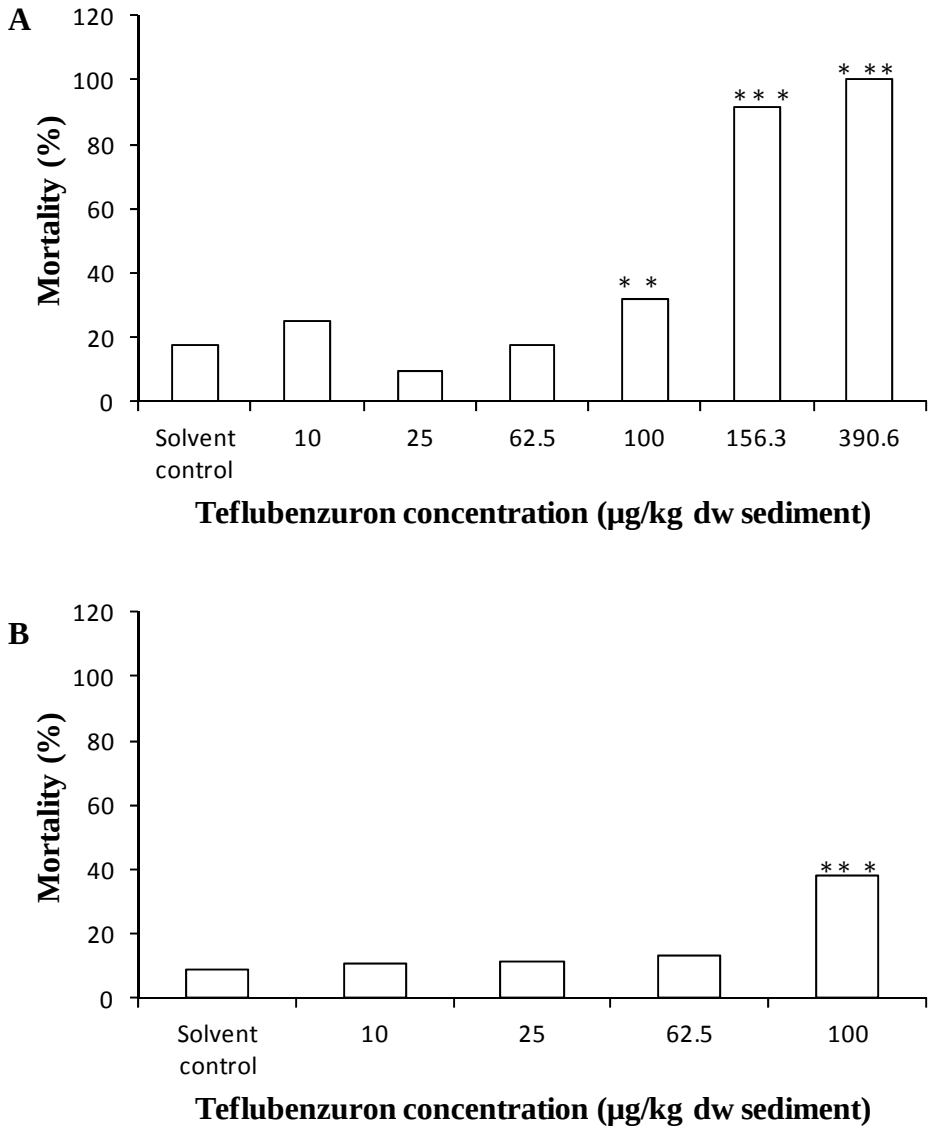

Fig. 2 Percentage of larval mortality of C. riparius during a 28-day static exposure (A) in the parental generation and (B) in the subsequent F1 generation. A chisquare test was used to compare treatments to the solvent control in each generation. Asterisks denote a significant difference (n=160; **p < 0.01; ***p < 0.001).

Development rate

In the P generation, the concentration of 62.5 µg/kg dw represented the NOEC compared to the solvent control while at 100 µg/kg dw (LOEC), significant adverse effects on development rate of midges have been observed compared to the solvent control (Fig. 3A). For the F1 generation, no adverse effects on development rate have been observed in treatments compared to solvent control (Fig. 3B).

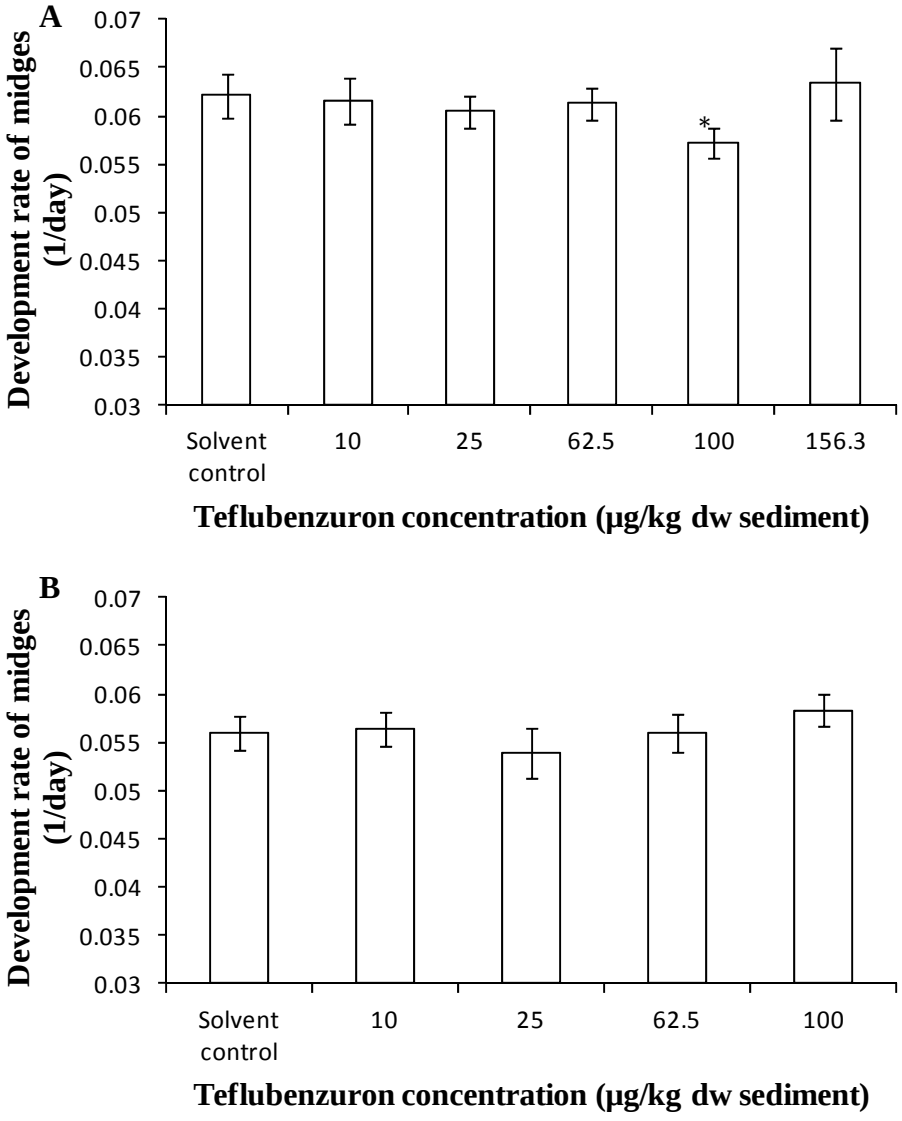

Fig. 3 Development rate (±SD, n = 8) of the P generation (A) and F1 generation (B) of *C. riparius* exposed to different teflubenzuron concentrations in sediment. Asterisks indicate a significant difference compared to the solvent control (Dunnett's test $p < 0.001$).

Sex ratio

The highest male fraction was about 80% at 156.3 µg/kg dw in the P generation but the data was not considered for statistical analysis since only 13 midges have been emerged from the 160 larvae inserted at the beginning of study. At 100 µg/kg dw, 62% of emerged midges were males. In the solvent control, the male fraction was about 54%. No significant differences were observed for the male fraction in any test concentration compared to the solvent control. The highest male fraction was about 59% at 100 µg/kg dw in the F1 generation and the lowest male fraction (46%) was observed at 25 µg/kg dw. In all other treatment groups, the male fraction was above 50% with a slight increase to 58% at 10 µg/kg dw. As for the P generation, no significant differences were detected for the male fraction, compared to the solvent control.

Fecundity of the P generation

Egg rope production by the females of the P generation is presented in Fig. 4. Most egg ropes were produced at 25 µg/kg dw (1.1 per female) and about 0.93 egg rope per female was produced in the solvent control. None of the females was able to lay egg ropes in the treatment of 156.3 µg/kg dw (Fig.4). An EC_{50} value of 112.7 µg/kg dw (95% CI, 82.05 and 143.4 µg/kg dw) was estimated (Weibull Type 2) for this endpoint.

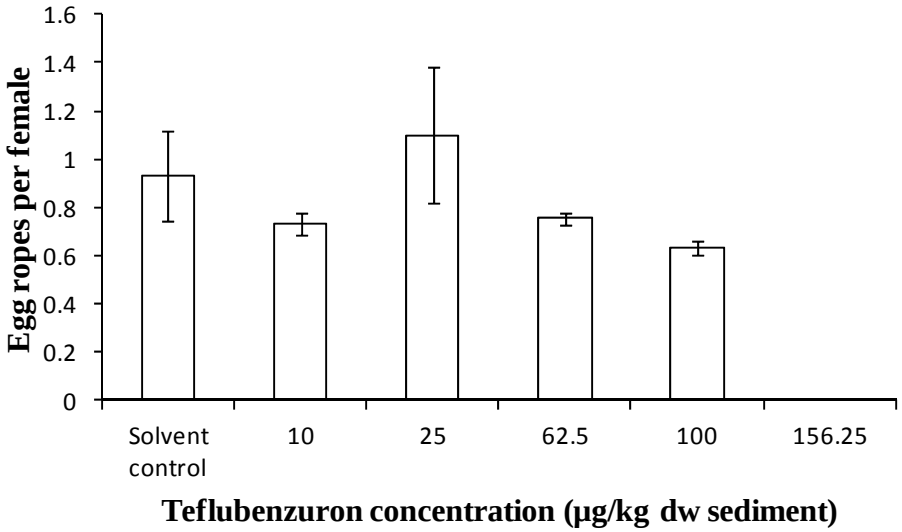

Fig. 4 The number of egg ropes per female (± SD, n = 8) produced by the P generation of C. riparius exposed to different teflubenzuron concentrations in sediment. An EC_{50} value of 112.7 mg/kg dw (95% CI, 82.05 and 143.4 µg /kg dw) was estimated (Weibull Type 2).

Fertility of egg ropes

A decrease in the fertility has been observed in all treatments compared to the solvent control (0.86) except the 25 µg/kg dw treatment in which the fertility was slightly elevated (0.97) (Fig.5). A dose-response analysis of the fertility data yields 74.5 µg/kg dw (95% CI, 40.1 and 108.9 µg/kg dw) as the EC_{50} concentration, at which fifty percent of the produced egg ropes were not able to hatch.

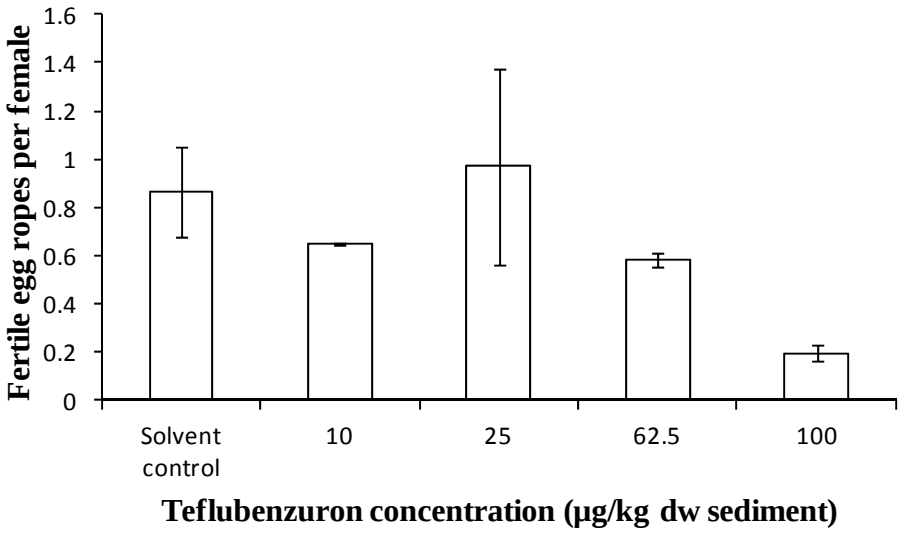

Fig. 5 Fertility (± SD, n = 8) of the P generation of C. riparius following static sediment spiked exposure with teflubenzuron showing a dose–response relationship with an EC_{50} value of 74.5 µg /kg dw (95% CI: 40.1–108.9 µg /kg dw).

Discussion

The present study over two-generation time indicates that exposure of *C. riparius* to the CSI teflubenzuron results in emerged midges affected in several aspects of their developmental and reproductive abilities. The data indicates that the emergence rate and to a certain extent also the development rate have been disrupted. NOEC values of 100 µg/kg dw for the P generation and 62.5 µg/kg dw for the F1 generation were estimated for the emergence rate, demonstrating that the F1 generation was more affected than the P generation. The differences in responses between the P and F1 generation were, however, minor from a quantitative point of view indicating the latency of effects of those compounds which might be explained by

their relatively high bioconcentration potential, affecting probably successive generations and thus leading to long-term ecological consequences. The results in the present study confirm our findings in previous works when first-instar larvae of the same species of *C. riparius* were exposed over two generations to another insect growth regulator pyriproxyfen (Tassou and Schulz, 2009). However, this is the first study showing teflubenzuron effects in a two-generation study using an aquatic organism. Campiche et al. (2006) stated that teflubenzuron was toxic for *F. candida* at concentrations that are probably close to environmental levels of this insecticide (toxicity/exposure ratios < 5). Teflubenzuron has also been reported to be highly effective in the reduction of adult emergence of the beetle *Callosobruchus maculatus* (Abo-Elghar et al. 2003). The reduction of the emergence of *C. riparius* in the present study may be a direct consequence of the mode of action of teflubenzuron as well as other benzoylureas compounds, which were considered as chitin synthesis inhibitors that act as anti-moulting agents, leading to the death of insect larvae and pupae as well as crustacaens (Haseeb and Amano, 2002; Cônsoli et al. 1998; SEPA, 1999; Mendez, 2006). To our knowledge, the precise mode of action of teflubenzuron and other chitin synthesis inhibitors (CSIs), is still unknown, however, three hypothetical target sites have been proposed, namely: inhibition of chitin synthetase (or its biosynthesis), inhibition of proteases (or its biosynthesis), and inhibition of UDP-N-acetylglucosamine transport through the membrane (Miyamoto et al. 1993). Mussian et al. (2009) reported that the direct target of benzoylureas compounds is not the chitin synthase itself, as these compounds fail to inhibit chitin synthesis in vitro. The authors suggested that benzoylureas may impede a sulfonylurea receptor (SUR)-related ABC transporter which may drive maturation of putative membrane compartments that are involved in chitin synthesis or influence the milieu of the extracellular space, where chitin fibres are deposited. Oetken et al. (2004), reported that although they interfere with moulting as an endocrine–regulated process in arthropods, the mechanism of CSIs action is purely non–endocrine. Because this synthesis usually takes place at or during the time of moulting, CSIs cause death during the moult, resembling the same effects as endocrine mimicking insect growth regulators. Inhibition of adult emergence due to application of CSIs (Novaluron, Triflumuron, Alsystin) to aquatic insects has been reported in recent studies by *Aedes aegypti, Anopheles stephensi*, *Culex quinquefasciatus*, *Culex culex pipiens* (Su et al. 2003; Batra et al. 2005; Martins et al. 2008; Rehimi and Soltani, 1999). Similar effects were also reported in aquatic mesocosm tests with diflubenzuron and lufenuron (Fairchild et al. 1996; Brock et al. 2009). Effects have also been observed for crustaceans which contain the

same moulting hormones as insects. For example diflubenzuron, the first CSI introduced into the market (Miyamoto et al. 1993) affected at ppm levels, the survival, larval development, regeneration and reproduction of macrocrustaceans (Nimmo et al. 1980; Ali and Mulla, 1978).

As to our knowledge, no comparable data illustrating two-generation tests with teflubenzuron are available; we suggest to perform studies over more than one generation to assess effects of sub-lethal teflubenzuron doses on non-target organisms' populations. Desneux et al. (2007) recommended to consider the sub-lethal effects of pesticides on arthropod physiology and behaviour for a complete analysis of pesticide impacts with the aim to determine if the evaluation of sub-lethal effects should be included in the registration process. Mèndez (2004, 2006) already recommended long-term investigations of the effects of teflubenzuron to assess its ecological relevance in sediments.

For the F1 generation no significant effect of teflubenzuron on development rate was observed at any test concentration compared to the solvent control. No comprehensive explanation can be given for the lack of significant effects on the development rate in the F1 generation, since the test duration (two generations) is most likely not long enough to assume a possible tolerance or resistance due to pre-exposure. Ristola et al. (2001) observed an ability of *C. riparius* to evolve tolerance to 2,4,5-trichlorophenol after three generations of pre-exposure time . A possible explanation can be that only the most resistant P-generation individuals reproduced and some maternal effects can also not be excluded. One hypothesis might probably be that compensation in the form of more food for surviving organisms have compensated the toxic effects (latent) by the F1 generation.

The fecundity of the P generation was one of the additional endpoints assessed by the present two-generation study compared to the standard test protocol of OECD 218. Our data indicate a decrease of the number of egg ropes per female in the treatments compared to the solvent control with an EC_{50} -value of 112.7 µg/kg dw, which is lower than the concentration of 156.3 µg/kg dw, at which no egg ropes were produced by females in the present study (Fig.4). Cônsoli et al. (1998) described a reduction of fecundity of the parasitoid *T. Pretiosum* when exposed to teflubenzuron or tebufenozide (an ecdysone agonist) before oogenesis. Relevant impacts of potential environmental concentrations of teflubenzuron on the reproduction rates of soil arthropods such as *F. candida* (Campiche et al. 2006) as well as the reduction in the oviposition of CSI-treated females of different insect orders have already been reported (Wilson and Cryan, 1997; Valle et al. 2009). Desneux et al. (2007) reported that reductions in

fecundity associated with pesticides may be due to both physiological and behavioural effects. The authors suggested also that insect growth regulators such as teflubenzuron may induce more long-term effects on fecundity than, for example, neurotoxins. Thus, the reduction of reproductive capacities of *C. riparius* in the present study may have biological implications for the ability to regulate their populations.

The fertility of the produced egg ropes was also assessed and as for the fecundity, our data indicate a decrease in egg hatch in treatments compared to solvent control (Fig.5). Hooper et al. (2005) observed the reduction of the number of egg ropes that hatched by 50% and more in *C. riparius* after sediment exposure to lufenuron. Martins et al. (2009) also observed less viable eggs due to exposition of *A. aegypti* to triflumuron. A decrease in egg hatch has also been observed in lacewing adults (Medina et al. 2002) as a result of diflubenzuron exposure.

Haseeb and Amano (2002) recorded reduction in parasitism, when some insect growth regulators (chlorfluazuron, flufenoxuron and teflubenzuron) were investigated by *C. plutellae* adults and suggested that these insecticides may affect oogenesis and the development of laid eggs. Another hypothesis of fertility reduction is that benzoylureas may impair the cuticle secretion in the affected embryo.

The present study does not indicate a sex related adverse effect of teflubenzuron on larvae in *C. riparius* from both P and F1 generations. No significant differences to solvent control were observed in any test concentration, although a highly significant difference in the proportion of males and females was noted in triflumuron-exposed group of *A. aegypti* compared to the controls (Valle et al. 2009). These results show that chemicals from the same group may not necessary cause the same effects in phylogenetic closely related species. Sex-related effects by *C. riparius* have already been observed in previous studies using biochemical methods (Hahn et al. 2001; Hahn and Schulz, 2002).

Conclusion

Since multigenerational experiments involve all sensitive life stages of an organism, they provide, as in the present study for teflubenzuron, additional data as useful amendment for the risk assessment but are very time consuming.

The effective concentration of teflubenzuron in the present study was above the environmental quality standard of 2.0 µg/kg dw sediment (SEPA, 1999). Test concentrations

in the present study can, however, also be considered environmentally relevant compared to observation by Mèndez (2006) with the cosmopolitan deposit-feeding polychaete *Capitella* species-complex. Campiche et al. (2006) also reported that teflubenzuron was toxic for *F. candida* at concentrations that are probably close to environmental levels of this insecticide (toxicity/exposure ratios < 5) with an EC_{50} value of 50 µg/kg dry weight after 28 day exposition time.

Our results suggest important biological implications as the reproductive parameters (fecundity and fertility) of *C. riparius* have been affected. Population-level consequences of considerable increases in the mortality of females could lead to reduced reproductive fitness and in consequence to a reduction in the abundance of future populations (Benoit et al. 1997), which finally may lead to genetic impoverishment. Desneux et al. (2006a) reported similar findings for the reproduction of *Aphidus ervi* (Hypmenoptera) and proposed to use the reproductive potential index for assessment of such reductions in reproduction.

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References

Abo-Elghar GE, El-Sheikh AE, El-Sayed FM, El-Maghraby HM, El-Zun HM. 2003. Persistence and residual activity of an organophosphate, pirimiphos-methyl, and three IGRs, hexaflumuron, teflubenzuron and pyriproxyfen, against the cowpea weevil, *Callosobruchus maculatus* (Coleoptera: Bruchidae). Pest Manag. Sci. 60:95-102.

Ali A, Mulla MS. 1978. Effects of Chironomid larvicides and diflubenzuron on Nontarget invertebrates in residential-recreational lakes. Environ. Entomol. 7:21-27.

Armitage P, Cranston PS, Pinder LCV. 1995. The Chironomidae, the biology and ecology of non-biting midges. Chapman and Hall, London.

Baird D. 1998. Expert Report Addendum. Report to Nutreco ARC June 1998.

Batra CP, Mittal PK, Adak T, Ansari MA. 2005. Efficacy of IGR compound Starycide 480 SC (triflumuron) against mosquito larvae in clear and polluted water. J. Vect. Borne Dis. 42:109-116.

Benoit DA, Sibley PK, Juenemann JL, Ankley GT. 1997. *Chironomus tentans* life-cycle test: design and evaluation for use in assessing toxicity of contaminated sediments. Environ. Toxicol. Chem. 16:1165-1176.

Brock CMT, Roessink I, Belgers JDM, Bransen F, Maund S J. 2009. Impact of a benzoyl urea insecticide on aquatic macroinvertebrates in ditch mesocosms with and without non-sprayed sections. Envrion. Toxicol. Chem. 28:2191-2205.

Campiche S, Becker-van Slooten K, Ridreau C, Tarradellas J. 2006. Effects of insects growth regulators on non target soil arthropod *Folsomia candida* (Collembola). Ecotox. Environ. Saf. 63:216-225.

Campiche S, L'Ambert G, Tarradellas J, Becker-van Slooten K. 2007. Multigeneration effects of insect growth regulators on the springtail *Folsomia candida*. Ecotox. Environ. Saf. 67:180-189.

Carbo L, Souza V, Dores EFGC, Ribero ML. 2008. Determination of pesticides multiresidues in shallow groundwater in a cotton-growing region of Mato Grosso, Brazil. J. Braz. Chem. Soc. 19:1111-1117.

Cônsoli FL, Parra JRP, Hassan SA. 1998. Side-effects of insecticides used in tomato fields on the egg parasitoid *Trichogramma pretiosum* Riley (Hym., Trichogrammatidae), a natural enemy of *Tuta absoluta* (Meyrick) (Lep., Gelechiidae). J. Appl. Ent. 122:43-47.

Desneux N, Denoyelle R, Kaiser L. 2006a. A multi-step bioassay to assess the effect of the deltamethrin on the parasitic wasp *Aphidius ervi*. Chemosphere. 65:1697-1706.

Desneux N, Ramirez-Romero R, Kaiser L. 2006b. Multi step bioassay to predict recolonization potential of emerging parasitoids after a pesticide treatment. Environ. Toxicol. Chem. 25:2675-2682.

Desneux N, Decourtye A, Delpuech JM. 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52:81-106.

Fairchild JF, Boyle TP, Robinson-Wilson E F, Haverland PS, Lebo JA. 1996. Ecological restructuring in experimental aquatic mesocosms due to the application of diflubenzuron. Environ. Toxicol. Chem. 15:1806-1814.

Mussian B, Gangishetti U, Breitenbach S, Zander M, Saheb SK, Müller U. 2009. Effects of benzoylphenylurea on chitin synthesis and orientation in the cuticle of the Drosophila larva. Eur. J. Cell Biol. 88:167-180.

Graf JF. 1993. The role of insect growth regulators in arthropod control. Parasitol. Today 9: 471-474.

Hahn T, Liess M, Schulz R. 2001. Effects of the hormone mimetic insecticide tebufenozide on *Chironomus riparius* larvae in two different exposure setups. Ecotoxicol. Environ. Saf. 49:171-178.

Hahn T, Schulz R. 2002. Ecdysteroid synthesis and imaginal disc development in the midge *Chironomus riparius* as biomarkers for endocrine effects of tributyltin Environ. Toxicol. Chem. 21:1052-1057.

Haseeb M, Amano H. 2002. Effects of contact, oral and persistent toxicity of selected pesticides on Cotesia plutellae (Hym., Braconidae), a potential parasitoid of *Plutella xylostella* (Lep., Plutellidae). J. Appl. Ent. 126:8-13.

Henrick CA, Staal GB, Siddall JB. 1973. Alkyl 3,7,11-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. J. Agric. Food Chem. 21:354-359.

Hooper HL, Sibly RM, Hutchinson TH, Maund SJ. 2005. Joint effects of density and a growth inhibitor on the life history and population growth rate of the midge *Chironomus riparius*. Environ. Toxicol. Chem. 24:1140-1145.

Ishaaya I, Casida JE. 1974. Diettary TH 6040 alters cuticle composition and enzyme activity of house fly larval cuticle. Pestic. Biochem. Physiol. 4:484-490.

Martins AJ, Belinato TA, Lima JB, Valle D. 2008. Chitin synthesis inhibitor effect on *Aedes aegypti* populations susceptible and resistance to organophosphate temephos. Pest Manag. Sci. 64:676-680.

Medina P, Smagghe G, Budia F, Estal P, Tirry L, Vinuela E. 2002. Significance of penetration, excretion, and transovarial uptake to toxicity of three insect growth regulators in predatory lacewing adults. Arch. Insect. Biochem. Physiol. 51:91-101.

Méndez N. 2005. Effects of teflubenzuron on larvae and juveniles of the polychaete *Capitella sp*. B from Barcelona, Spain. Water, Air, Soil Pollut. 160, 259-269.

Méndez N. 2006. Effects of teflubenzuron on sediment processing by members of the *Capitella* species-complex. Environ. Pollut. 139:118-124.

Miyamoto J, Hirano M, Takimoto Y, Hatakoshi M. 1993. Insect growth regulators for pest control, with emphasis on juvenile hormone analogs: Present status and future prospects. ACS Symp. Ser., ACS, Washington, DC. 524:144-168.

Mondal K, Parween S. 2000. Insect growth regulators and their potential in the management of stored-product insect pests. Int. Pest. Manag. Rev. 5:255-295.

Mulla MS, Thavara U, Tawatsin A, Chompoosri J, Zaim M, Su T. 2003. Laborabotory and field evaluation of novaluron, a new acylurea insect growth regulator, against *Aedes aegypti* (Diptera: Culicidea). J. Vec. Ecol. 28:241-254.

Nimmo DR, Hamaker TL, Moore JC, Wood RA. 1980. Acute and chronic effects of dimilin on survival and reproduction of Mysidopsis bahia, In: Aquatic Toxicology, ASTM STP 707 (Eds. Eaton, J.G., Parrish, P.R., Hendricks, E.C.,). American Society for Testing and Materials, Philadelphia, PA. Pp. 366-376.

OECD 2004. Guideline for the testing of chemicals, sediment-water Chironomid toxicity test using spiked sediment, OECD 218, adopted 13 April 2004.

OECD 2006. Detailed review paper on aquatic arthropods in life cycle toxicity tests with an emphasis on developmental, reproductive and endocrine disruptive effects. OECD document, No 55, Organisation for Economic Co-operation and Development, Paris, France.

Oetken M, Bachmann J, Schulte-Oehlmann U, Oehlmann J. 2004. Evidence for endocrine disruption in invertebrates. Int. Rev. Cytol. 236:1–44.

Pennak R. 1989. Fresh-water invertebrates of the United States. Wiley,New York.

Post LC, de Yong BJ, Vincent WR. 1974. 1-(2,6-disubstitued benzoyl)-3-phenylurea insecticides: inhibitors of chitin synthesis. Pestic. Biochem. Physiol. 4:473-483.

Rasmussen JB. 1984. The life-history, distribution, and production of *Chironomus riparius* and *Glyptotendipes paripes* in a prairie pond. Hydrobiologia 119:65-72.

Ristola T, Parker D, Kukkonen JVK. 2001. Life cycle effects of sediment-associated 2,4,5-trichlorophenol on two groups of the midge *Chironomus riparius* with different exposure histories. Environ. Toxicol. Chem., 20 (8), 1772-1777.

Reynolds SE. 1987. The cuticle, growth and moulting in insects:the essential background to action of acylurea insecticides. Pest. Sci. 20, 131-146.

SEPA 1999. Policy No. 29. Calicide (teflubenzuron) - Authorisation for use as an in-feed sea lice treatment in marine cage salmon farms. Risk Assessment, EQS and Recommandations, Final Report, July, 1999.

Scott Brown SA, Simmonds SJM, Blaney MW. 2003. Influence of a short exposure to teflubenzuron residues on the predation of thrips by *Iphiseius degenerans* (Acari: Phytoseiidae) and *Orius laevigatus* (Hemiptera: Anthocoridae). Pest. Manag. Sci. 59: 1255-1259.

Soltani N, Rehimi N. 1999. Laboratory evaluation of alsystin, a chitin synthesis inhibitor, against *Culex pipiens pipiens* L. (Diptera: Culicidae): effects on development and cuticle secretion. J. Appl. Ent. 123:437-441.

Stark JD, Banks JE. 2003. Population-level effects of pesticides and other toxicants on arthropods. Annu. Rev. Entomol. 48:505-519.

Su T, Mulla MS, Zaim M. 2003. Laboratory and field evaluations of novaluron, a new insect growth regulator (IGR), against *Culex* mosquitoes. J. Am. Mosq. Control Assoc. 19: 408-418.

Sundaram KMS, Holmes SB, Kreutzweiser DP, Sundaram A, Kingsbury PD. 1991. Environmental persistence and impact of diflubenzuron in a forest aquatic environment following aerial application. Arch. Environ. Contam. Toxicol. 20:313-324.

Taenzler V, Bruns E, Dorgerloh M, Pfeifle V, Weltje L. 2007. Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. Ecotoxicology, 16:221-230.

Tassou KT, Schulz R. 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotoxicol. Environ. Saf. 72:1058-1062.

Trouw 1999. Additional Reports on Environmental Safety of Calicide. Report to SEPA by Trouw Aquaculture ARC, 2 volumes March 1999.

Tunaz H, Uygun N. 2004. Insect growth regulators for insect pest control. Turk. J. Agric. For. 28:377-387.

Valle D, Belinato TA, Martins AJ, Lima JB, de Lima-Camara TN, Peixoto AA. 2009. Effect of the chitin synthesis inhibitor triflumuron on the development, viability and reproduction of Aedes aegypti. Mem. Inst. Oswaldo Cruz, Rio de Janeiro 104:43-47.

Watts MM, Pascoe D, Carroll K. 2001. Chronic exposure to 17 alpha-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diptera: Chironomidae). Aquat. Toxicol. 55:13-24.

Wilson TG, Cryan JR. 1997. Lufenuron, a chitin-synthesis inhibitor, interrupts development of *Drosophila melanogaster*. J. Exp. Zool. 278:37-44.

Appendix II

Low field-relevant tebufenozide concentrations affect reproduction in *Chironomus riparius* (Diptera: Chironomidae) in a long-term toxicity test

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Abstract

A few studies reporting the effects of tebufenozide, a non-steroidal ecdysone agonist that mimics natural moulting hormones in Chironomids exist in the literature. However, nothing is known about its chronic or multigenerational effects on the reproduction of aquatic insects, although tebufenozide is present in aquatic ecosystems. Here, we investigated the chronic toxicity of tebufenozide in two successive generations of *Chironomus riparius* using nominal concentrations that ranged from 4 to 26.2 µg/L. We started the test from the first instar larvae in the parental (P) generation, quantifying life cycle parameters (emergence, sex ratio, development rate, fecundity and fertility) until the emergence in the subsequent F1 generation. Results showed a reduction in reproduction and a significant decrease in male developmental rate of midges for all treatments, in the F1 generation compared to the P generation (paired t-test; $p < 0.001$). Two-way analysis of variance revealed a significant exposure × generation effect on male fraction with male fraction increasing (P generation) or decreasing (F1 generation) with increasing exposure. These effects on *C. riparius* underline the importance of conducting long-term studies with environmentally relevant concentrations to investigate population-level endpoints for endocrine disrupting chemicals.

Keywords: Tebufenozide; *Chironomus riparius*; Life-cycle Test; reproduction; population-level effects; endocrine disruption.

Introduction

Two of the key insect hormones that are relevant to the evaluation of endocrine disrupting chemicals (EDCs) are ecdysteroids and juvenile hormones (Kropp et al. 2004). Insect growth regulators (IGRs) represent a group of insecticides. They are known as the third generation of insecticides, which were developed intentionally to mimic, block or otherwise interact with the hormonal system of insects (Oetken et al. 2004). These include juvenile hormone analogues, chitin synthesis inhibitors (CSIs), and ecdysone agonists (Tunaz and Uygun 2004). Ecdysone agonists are benzoyl hydrazines which have been reported to act as agonists of ecdysteroidal moulting hormone at the molecular level and, therefore, cause a variety of hormonal effects in insects and crustacean arthropods (Graham et al. 2007; Tarrant et al. 2011; Yokota et al. 2011).

Tebufenozide is a moult-inducing insecticide that has been developed for the control of lepidopteran pests in agriculture including forest and fruit-crops. This compound is a non-steroidal ecdysone agonist that mimics natural moulting hormones, which principally contains 20-OH ecdysone in larval insect. Insecticidal activity of tebufenozide is shown by inducing premature and incomplete larval moult (Wing et al. 1988). Based on its specificity towards lepidopteran insects, tebufenozide is considered a compound with high environmental safety (Sundaram et al. 1998). Tebufenozide has been found regularly in surface water discharging from a fruit orchard area in Germany at levels of up to 20 µg/L (Süß et al. 2006). It was the most frequently detected compound found following its application, in 59% of the total of 406 analyzed samples over extended time periods in surface water (Süß et al. 2006).

Though tebufenozide has been suspected to persist at rather low concentrations in the aquatic ecosystems (Sandaram 1997; Süß et al. 2006), most toxicity tests with this compound, until now, were carried out on non-target aquatic arthropods using high concentrations to make effects visible. Kreutzweiser et al. (1994) observed non-significant effects on downstream drift or survival of 11 species of aquatic insects in the laboratory following flow through toxicity tests with tebufenozide at 3.5 mg/L. Song et al. (1997) observed tolerance to tebufenozide concentrations as high as 10 mg/L and 2.5 mg/L at 20°C in two freshwater species *Daphnia magna* and *Aedes aegypti* respectively. However, it is important to note that those studies primarily referred to acute test durations with mortality as an endpoint and effective concentrations well above field relevant levels.

Based on our research in the web of science database, only Hahn et al. (2001) reported NOEC and LOEC values of 13.2 and 17.2 µg/L respectively for *C. riparius* during a 24-day toxicity test after static contamination of first instar larvae with tebufenozide. A chronic study with Chironomids had not yet been conducted. A 21-day test with NOEC value of 29 µg/L tebufenozide was reported from a flow-through study with *D. magna* in Canada (Pest Management Regulatory Agency 1996). These effect thresholds demonstrated much lower limits for lethal effects of tebufenozide in aquatic arthropods and provided a hint to the fact that long-term tests of chronic toxicity with EDCs may yield valuable additional information to improve upon the standard risk assessment.

Despite its frequent detection in surface water and its persistence in aquatic environments, no study, until now has assessed the effects of tebufenozide on the reproduction of an aquatic insect or investigated its long-term effects over more than one generation in freshwater organisms.

Hence, the aims of the present study was to assess the effects of tebufenozide on the reproduction of an aquatic insect and to investigate long-term effects of its environmentally relevant concentrations over two generations on life cycle parameters of *C. riparius*. This may complement the current risk assessment and contribute to the characterization and detection of EDCs endpoints in aquatic insects, considering the important role that aquatic insects play in nutrient processing, storage or cycling.

Materials and methods

Test insect

Chironomus riparius (Diptera: Chironomidae) was used in this full life cycle test over two generations. Egg ropes used to conduct the present study were from our in-house laboratory culture, established with egg ropes that were originally obtained from BASF SE, Limburgerhof. Midges were reared in a SANYO chamber under long day conditions at 20 °C with a 16:8 h light/dark regime with a light intensity of 708±50 lux. One hour dusk phase was also generated for the mating of the adult animals in the breeding cage. A relative air humidity of 75% ± 5% was maintained throughout the rearing time.

Test insecticide

Tebufenozide (N-ter-butyl-N'-[4-ethyl-benzoyl]-3,5-dimethylbenzohydrazide, formerly RH-5992) Pestanal (99.9%) was purchased from Sigma-Aldrich Chemie, Steinheim (Germany). The compound is a white powder, that has a low water solubility of 0.83 mg/L at 20°C and a high water:octanol partition coefficient (log Kow = 4.25) at pH 7 and 20°C. It belongs to a group of IGRs known as benzoyl hydrazines, which have been extensively studied. Tebufenozide is commercially known under the names Confirm®, Romdan®, and Mimic® (Dhadialla et al. 1998) for use in rice fields in Southeast Asia, orchards, forestry, and agriculture in Canada and the United States. In Europe it is used in vineyards and apple orchards. For the study, a stock solution was prepared in dimethylsulfoxid (DMSO 99.8%) with a nominal concentration of 0.5 mg/L active ingredient. From this stock solution, test solutions of the chosen concentrations were prepared by dilution with DMSO. Aliquots of 33.6 µL of each test solution were placed in the respective test vessels, resulting in nominal test concentrations from 4 to 26.2 µg active ingredient/L tebufenozide.

General test method

The test method largely follows that described in Tassou and Schulz (2011) with the difference that exposure design was spiked water. M4-medium was chosen as test water. Stock solutions and water spiking processes have been done according to OECD guideline 219 (OECD 2004). Each test vessel (600 mL glass beaker) contained 400 mL M4-medium and 100 g moist artificial standard non sterilized sediment with a pH of 7.0 ± 0.5 according to OECD 2004. The sediment-water system was gently aerated (2 bubbles per second) for seven days, following setup of the test vessels, and prior to insertion of 20 first instar larvae of the P generation. Following addition of the larvae, aeration was interrupted for 24 h to enable the larvae to settle within the sediment. The first instar larvae were exposed to five nominal test concentrations (4, 6.4, 10.2, 16.4, and 26.2 µg/L) of tebufenozide, including one solvent control with DMSO at a level equivalent to the highest DMSO concentration (84 µL/L) present in the tebufenozide treatments. Eight replicates were used for each test concentration, as well as the solvent control under static conditions. Twenty-four hours after adding the first instar larvae to the test vessels of the P generation, test solutions previously prepared were added to the water overlying the sediment using a pipette. For the F1 generation, larvae were transferred directly into vessels containing spiked water and prepared as described above. The

overlying water was then mixed to ensure homogeneous distribution while taking care not to upset the sediment. The test concentrations were estimated on the basis of the water overlying the sediment. The larvae were fed daily with commercial fish-food (Tetra Min®) according to the method provided in Tassou and Schulz (2011). The adult midges were transferred with an exhauster into two breeding cages per treatment (50 x 50 x 50 cm), where they could swarm, mate, and oviposit into a 2-L glass crystallization dish filled with 1 L non-aerated M4-medium spiked with the respective tebufenozide levels and 300 g formulated sediment. The egg ropes were collected daily from the crystallization dishes and kept for observation in 12-well microtiter plates containing spiked water from the respective dish to assess the reproduction. To start the F1 generation, six fertile egg ropes from one or two consecutive days around day 19 of the P generation test phase were selected from each breeding cage. After hatching, 20 first instar larvae were allocated randomly to each of the newly prepared test vessels for the F1 generation test phase as mentioned above. Both the P and F1 generation were each investigated separately for a 28–day test period. The effect of tebufenozide on the reproduction of the P generation was assessed and the development rate, the emergence ratio of fully emerged midges, and their sexes were determined over the two generations. The test was conducted in a climate chamber at 20°C and 70±10% relative air humidity as described in Tassou and Schulz (2011).

Chemical analysis

Samples of the water overlying the sediment and pore water were analysed from additional test vessels at the start and the end of the test for the P and F1 generation. Centrifugation at 10000 g and 4°C for 30 minutes was proceeded to extract pore water from the sediment. Water samples (overlying water and pore-water) were then immediately frozen at -20°C until analysis by an Exactive LC-MS instrument equipped with a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland), an Accela pump, and a Surveyor LC pump following online solid-phase extraction using an Equan System (ThermoFisher Scientific, Dreieich, Germany). Water purified with a MiliQ system (Millipore, Schwalbach, Germany) containing 0.1% formic acid and 4 mmol ammonium formate (both Sigma Aldrich, Seelze, Germany, puriss. p.a. grade) (solvent A) and acetonitril (hypergrade, Merck, Darmstadt, Germany) containing 0.1% formic acid and 4 mmol ammonium formate (solvent B) were used as eluents. 500 µL of the aqueous sample were injected on the EQuan system equipped with a Hypersil Gold aQ column (20 × 2.1 mm; particle size 12 µm) for concentration with a flow

rate of 2 mL/min of 98% solvent B and 2% solvent A. The sample was transferred on-line to the analytical column Hypersil Gold C18 column purchased from Thermo Fisher Scientific (50 × 2.1 mm, particle size 1.9 µm) with a flow rate of 200 µL/min. The gradient started with 95% A and 5% B for 2 minutes, and was raised to 100% B in 2 minutes and held for 4 min. The analytical column was conditioned for 2.5 min with 95% A and 5% B. The analytes were ionized using an Ion Max API Source with an ESI probe operated at room temperature with a spray voltage of 3.6 kV. Nitrogen was used as sheet gas and auxiliary gas with flow rates of 20 mL/min and 5 mL/min, respectively. The ions were detected in the scan range of m/z 100-2000 in the positive mode. The compound was identified using the accurate mass of the $[M+H]^+$ with deviations always smaller than 5 ppm between theoretical and measured mass. For quantification the $[M+H]^+$ together with an external calibration were used. The results are reported in Table 1 with a limit of detection of 2.5 ng/L.

Table 1: Nominal and measured concentrations of tebufenozide for the P and F1 generation

		Measured concentrations (µg/L)			
		P Generation		F1 Generation	
Sample	**Nominal concentrations (µg/L)**				
		Day 0	Day 28	Day 0	Day 28
Overlying water	Solvent control	< LOD	< LOD	< LOD	< LOD
	4	1.6	0.29	2.8	0.5
	6.4	6.4	1.08	5.8	1.1
	10.2	5.47	1.13	10.0	2.3
	16.4	10.07	2.72	14.5	2.7
	26.2	21.6	3.47		
Pore-water	Solvent control	< LOD	< LOD	< LOD	< LOD
	4	< LOD	0.09	< LOD	0.1
	6.4	< LOD	0.28	< LOD	0.3
	10.2	0.06	0.31	0.4	0.7
	16.4	0.19	0.61	0.3	1.1
	26.2	0.5	1.7		

LOD: limit of detection (2.5 ng/L)

Data analysis

The emergence rate data for both P and F1 generations were arcsin-sqrt transformed to obtain a normal distribution and to improve variance homogeneity. The statistical evaluation was conducted using the software package SPSS 16.0 for Windows ®. ANOVA, followed by Dunnett's test, was used for this endpoint to compare the treatment groups with the solvent control. The level of significance was set at 95% ($\alpha = 0.05$). For the evaluation of sex ratio

data, Fisher's exact test (with Bonferroni adjustment) was used. Additionally, factorial analysis of variance was performed to evaluate potential interaction effect of exposure (control vs. treatments) and generation (P vs. F1) on the male fraction. Paired samples test were conducted to check for differences in the male development rate between P and F1 generation. For the reproduction, a dose-response relationship was estimated with the log-logistic regression model (Weibull Type 2).

Results

Emergence ratio

At the chosen test concentrations, no effect on the emergence of *C. riparius* has been detected for the P and F1 generation. The emergence ratio in the P generation even at the highest test concentration of 26.2 µg/L was over 80%. In the F1 generation, the emergence ratio of midges at 16.4 µg/L, which represents the highest concentration for this generation, was 91.25%. The criterion for validity of 70% of emerged adult midges within the solvent control (OECD guideline 233) was fulfilled with 89.4% and 89.2% emergence ratio in the P and F1 generation, respectively.

Development rate

No effects were observed on the overall development rate even at the highest concentration tested of 26.2 µg/L in the P and 16.4 µg/L in the F1 generation. However, the male development rate was significantly (paired sampled test $p < 0.001$) lower in the F1 generation compared to the P generation in the tebufenozide exposure setups (Fig. 1).

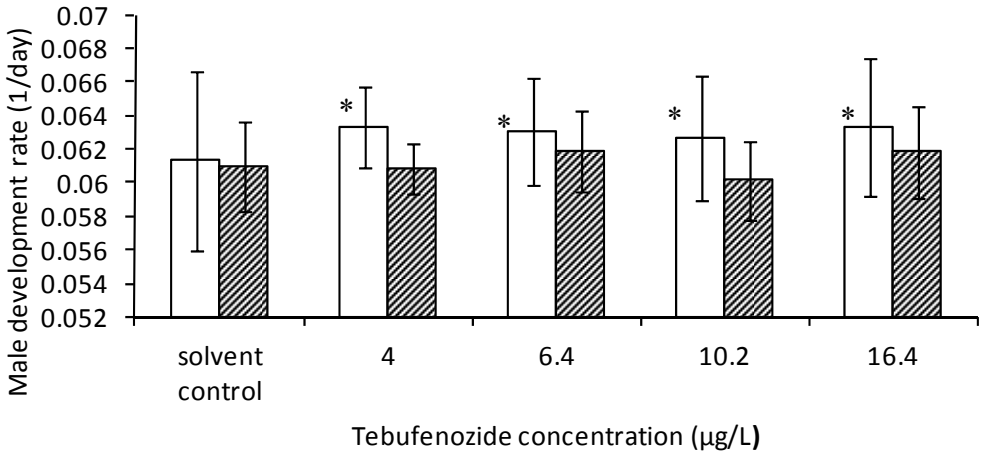

Fig. 1 Mean (± standard deviation, n = 8) development rate of males of *C. riparius* after static exposure to tebufenozide over two generations. The male development rate in the tebufenozide treatments of the P generation (white bars) was significantly higher (paired t-test; $p < 0.001$) than those of the F1 generation (hatched bars) and denotes with asterisks.

Sex ratio

No statistically significant difference in sex ratio, expressed here as the male fraction, was present between the control and other treatments for the P generation, even if the male fraction increased with increasing test concentrations (Fig. 2 A). For the F1 generation, a significantly elevated male fraction was found in the 4 µg/L treatment and a trend to decrease in the male fraction was present with increasing test concentrations (Fig. 2 B).

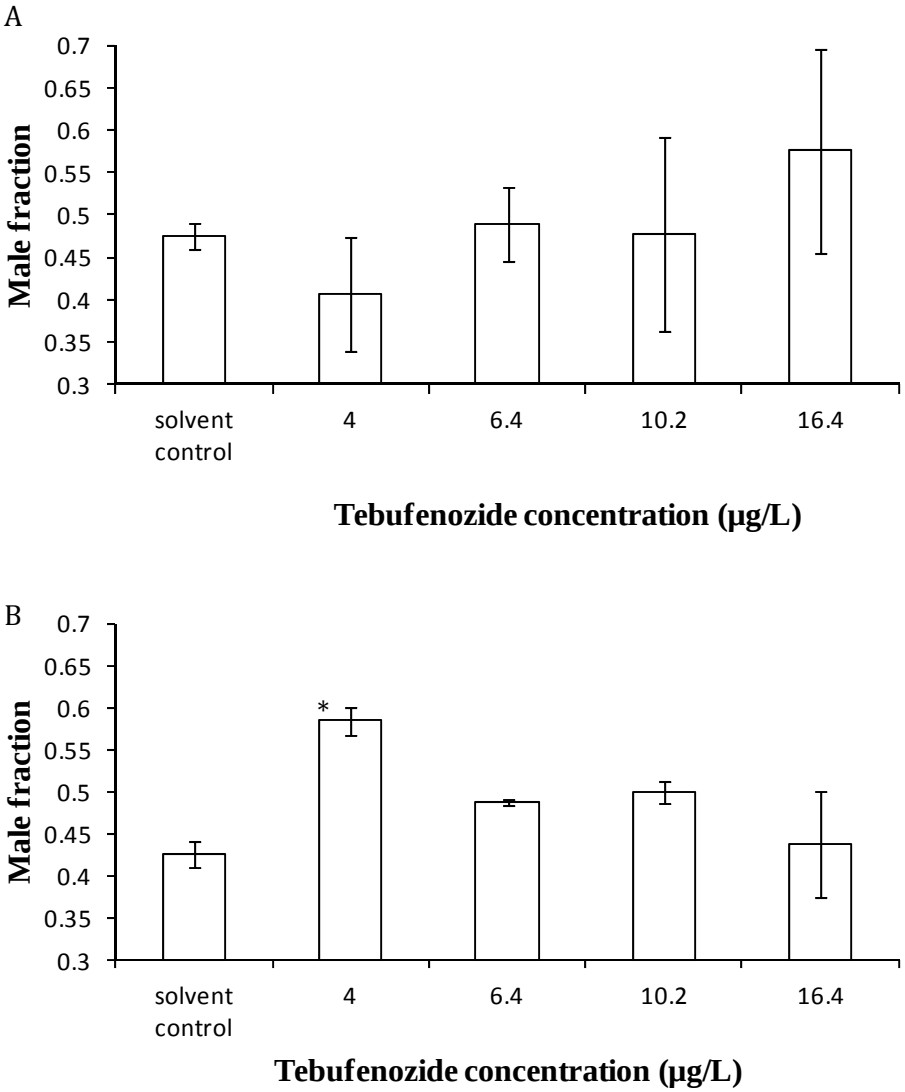

Fig. 2 Mean male fraction (± standard deviation, n = 8) of *C. riparius* after static exposure to tebufenozide over two generations (A: P generation; B: F1 generation). Asterisk denotes a significant difference compared to the solvent control (Fisher's Exact Test, p = 0.009).

Factorial analysis of variance indicated a significant (p = 0.004) interaction effect of exposure (control vs. tebufenozide treatments) and generation (P vs. F1) on male fraction of *C. riparius* (Table 2). Both exposure and generation alone did not show any significant effect.

Table 2: Factorial analysis of variance of the effect of exposure (control vs. tebufenozide treatments) and generation (P vs. F1) on the male fraction of *C. riparius*.

	Source	**df**	**MS**	**F**	**p**
Male fraction	Exposure	3	0.001	0.083	0.969
	Generation	1	0.004	0.311	0.579
	Exposure × Generation	3	0.066	4.935	0.004
	Error	56	0.013		

df = degrees of freedom; MS = mean square; F = likelihood ratio; p = probability

Fecundity of the P generation

Egg rope production by the females of the P generation is presented in Fig. 3 A. Most egg ropes (1.03 per female) were produced at 10.2 µg/L and least (0.34 per female) were found in the highest test concentration of 26.2 µg/L. An EC_{50}-value of 23.8 µg/L (95% CI, 19.1 and 26.9 µg/L) was estimated (Weibull Type 2) for this endpoint.

Fertility of egg ropes of the P generation

No differences in fertility were detected in treatments compared to the solvent control (Fig. 3 B), except for the highest test concentration of 26.2 µg/L, which showed a significant decrease in fertility compared to the solvent control. The fertility in this concentration was also extended over days with only one or two fertile egg ropes per day. It was therefore not possible to conduct the second generation with this concentration of 26.2 µg/L. A dose-response effect investigation on the fertility gives 23.0 µg/L (95% CI, 19.1 and 26.9 µg/L) as the concentration at which fifty percent of the produced egg ropes were not able to hatch.

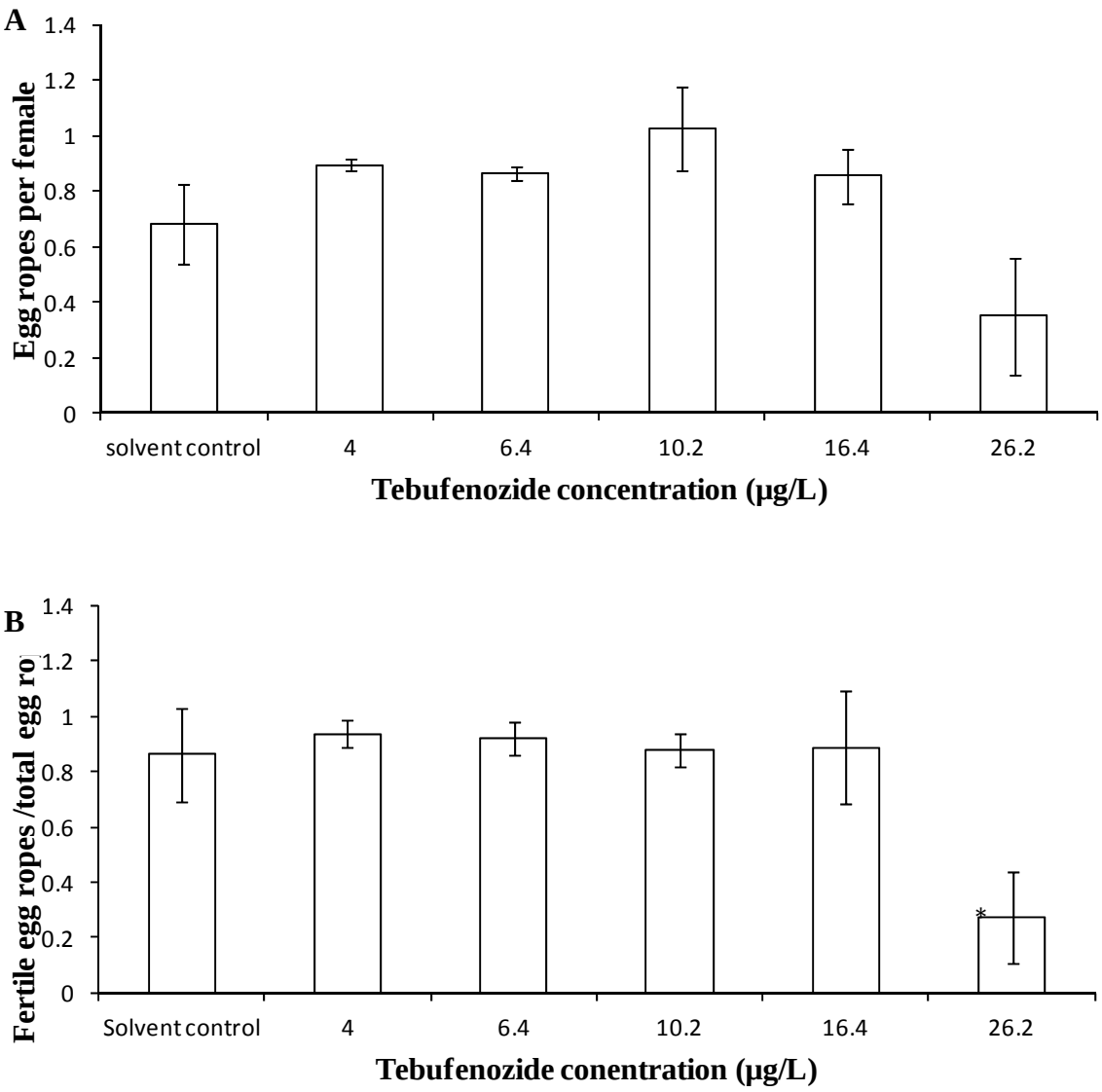

Fig. 3 Effects of sub-lethal tebufenozide concentrations on the reproduction of the P generation of *C. riparius* (A: fecundity; B: fertility) after a 28-days static exposure of first instar larvae. Asterisk denotes a significant difference compared to the solvent control (ANOVA, Dunnett's test $p = 0.014$).

Discussion

The present two-generation study indicated that exposure to environmentally relevant sub-lethal concentrations of tebufenozide affected developmental and reproductive processes of *C. riparius*. The results indicated a reduction in reproduction (Fig. 3) and emphasised the

importance of considering reproduction as an endpoint for the detection of EDCs. This is important considering the role that reproduction plays as the main process linking the individual to the population. This is also an additional endpoint suggested in the OECD guideline 233 compared to the standard chronic *Chironomus* study (OECD guidelines 2004). To our knowledge, this is the first study reporting effects of tebufenozide on reproduction in an aquatic insect although the compound has been suspected to be persistent in aquatic ecosystems (Sundaram 1997; Süß et al. 2006). The present study therefore contributes to a better characterization and risk assessment of tebufenozide in the aquatic environments.

Tebufenozide effects on the reproduction have however, been reported in the literature for other insect orders. Cônsoli et al. (1998) for example, reported a reduction of fecundity of the parasitoid *Trichogramma pretiosum* following exposure to tebufenozide before oogenesis. Some studies reporting sub-lethal effects of tebufenozide and other ecdysteroid agonists on the reproduction of pest species from several terrestrial insect orders are available (Sun et al. 2000; Smagghe et al. 2004). Smagghe and Degheele (1997) observed an inhibition of oviposition after treatment of larval stages of *Spodoptera littoralis* with tebufenozide.

The resorption of oocytes by females and the production of smaller ovaries or eggs with abnormal chorion development were reported in the literature as possible inhibitory effects of non-steroidal ecdysteroids in several insect species. Sun et al. (2003) suggested that tebufenozide may regulate the vitellogenin synthesis in females via the ecdysteroid receptor protein complex.

Our data for fecundity showed a dose-response relationship, with an EC_{50}-value of 23.76 µg/L. This is lower than the concentration at which the lowest number of egg ropes was produced. The EC_{50}-value of 23 µg/L estimated for the fertility was also lower than the concentration of 26.2 µg/L that showed a decrease in egg hatch rates ($p = 0.014$; Fig.3 B). Thus, the selected concentrations as well as the concentrations showing effects in the present study are environmentally relevant since Kreutzweiser et al. (1998) expected a maximum aqueous concentration of 52 µg/L tebufenozide in a mesocosm study after forestry applications. The highest concentration (26.2 µg/L) used in the present study is twice as low as the maximum expected field concentration. This is also in the range of the frequently detected concentration of 20 µg/L tebufenozide in surface water in Germany over extended time periods following its application in orchards (Süß et al. 2006). Hence this study

underlines the importance of investigating long-term effects of sub-lethal tebufenozide concentrations on non-target aquatic insects' populations that are ecologically important.

The observed adverse effects of tebufenozide on the reproduction of *C. riparius* indicated that tebufenozide might control populations of *C. riparius* once it reached the aquatic ecosystem. This emphasised also that tebufenozide might exhibit a significant effect on the population dynamics of *C. riparius*. Considering its half-life of 40 days (Sundaram 1997), tebufenozide may be crucial for the offspring of *C. riparius*, and hence might impede its population sustainability. Biddinger et al. (2006) also reported a significant effect on the population dynamics of a field strain of the Lepidoptera *Platynota idaeusalis*, however only when exposed to tebufenozide at ppm levels. Additionally, Cadogan et al. (2002) suggested that the presence of tebufenozide in the environment may have multiple year carry-over effects with a significant reduction in populations of the Lepidoptera *Choristoneura fumiferana*.

The observed reduction in the male developmental rate of treated midges in the F1 generation ($p < 0.001$) compared to the P generation (Fig. 1) might indicate an increase in sensitivity of the second generation due to accumulation or carry-over effects. This could also be explained by an increased affinity of tebufenozide (a non-steroidal ecdysone agonist) for the ecdysone receptor complex that is primarily responsible for increased toxicity to *Chironomus* larvae (Smagghe et al. 2002) as a result of the extension of the exposure to the second generation. This is supposedly the case since there was no difference in the male development rate of the controls between both P and F1 generations (Fig. 1). This elevated sensitivity of the F1 generation is in accordance with previous studies, when *C. riparius* was exposed over two generations to other IGRs (Taenzler et al. 2007; Tassou and Schulz 2009, 2011) and is highly relevant to the risk assessment for chemicals with endocrine disruptive potential.

For the first time, a significant two-way interaction of exposure × generation ($p < 0.004$) on the male fraction in an aquatic insect was found following extended exposure over two generations, while both exposure and generation alone did not indicate any significance (Table 2). In particular, the male fraction was significantly elevated in the lowest tebufenozide treatment only in the F1 generation. This observation, that the greater effect is associated with exposure to lower concentrations has previously been reported for EDCs whose behaviour may not necessarily be adequately characterized by the classic dose-response curves (Santillo et al. 1998).

The present study thus underlines the necessity of conducting studies over more than one generation to assess the reproductive phase of test organisms and to make effects of persistent chemicals or EDCs, which might not be observed through conventional acute or chronic testing, visible. Tests over several generations can be helpful in identifying and detecting effects at low levels and hence add valuable information to the risk assessment at the population level. The effects on male developmental rate, as observed in the present study (Fig. 1), are likely to be due to the disruption of hormonal processes directly or indirectly controlled by ecdysteroids (Kwak and Lee 2005). Tebufenozide is mimicking the natural moulting hormones which principally contain 20-OH ecdysone by binding competitively to ecdysteroid receptors in insect cells (Dhadialla et al. 1998). Moreover, the enzyme systems involved in the cytochrome P450 monoxygenases may also have been affected by tebufenozide exposure suggesting that more than one mode of action can be expected with regard to the effects of tebufenozide in *C. plumosus* (Kwak and Lee 2005). This assertion may explain the behaviour of tebufenozide over the present two-generation test, or the incorporation of mechanistically linked endpoints in the present test design is needed to thoroughly investigate the long-term effects of low field-relevant tebufenozide concentrations on *C. riparius*. The unexpected pattern of the male fraction observed in the F1 generation compared to the P generation (Fig. 2), although tebufenozide may induce symptoms of hyperecdysonism (Soin and Smagghe 2007), might support the above mentioned assertion of other authors about its mode of action. The occurrence of ecdysone receptor isoforms within species and even within the tissues and cells of a single specimen may at least partially explain the differences among arthropods in susceptibility to tebufenozide contamination (Lezzi et al. 1999).

Hahn et al. (2001) reported for *C. riparius* and tebufenozide that pupal mortality was twice as high in males as in females during a 100 µg/L treatment over an 8 day test duration with the contamination starting on day 12 post hatching. In contrast, the present study revealed a significant effect on the male fraction already at about a 25 times lower tebufenozide concentration of 4 µg/L as a result of an extended exposure. Reynolds et al. (2004) also reported that larval exposure to sub-lethal doses of tebufenozide significantly impairs subsequent male reproduction function in the Lepidoptera *Spodoptera litura* with effects manifested at lower ppm levels (0.5-2 ppm) of the insecticide. These authors explained their observation as the result of a direct interference by residual insecticide with ecdysteroid-

dependent testicular development and spermatogenesis in males, or even indirect interference with juvenile hormone-controlled processes, as the actions of ecdysteroids and juvenile hormones are closely interlinked.

Conclusion

The present study assessed for the first time the effects of tebufenozide on the reproduction of an aquatic insect *C. riparius*. This is also the first study investigating long-term exposure of tebufenozide over two generation in the aquatic environments. Results provided population-relevant information (reduction in reproduction, decrease in developmental rate in the second generation) that can be used for a better characterization and risk assessment of tebufenozide in the aquatic ecosystems. In addition, the fluctuation of the male fraction observed here with different patterns in the two generations, requires further attention, since it can play an important role for the sustainability of populations. This study further shows the susceptibility of *C. riparius* in detecting hormonally adverse effects at low environmentally relevant concentrations. It contributes to understanding and detecting endpoints for EDCs including not only the reproductive phase of the test species but also taking all relevant life stages and effects in two successive generations into account.

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References

Biddinger D, Hull L, Huang H, McPheron B, Loyer M. 2006. Sublethal effects of chronic exposure to tebufenozide on the development, survival, and reproduction of the tufted apple bud moth (Lepidoptera: Tortricidae). J. Econ. Entomol. 99: 834-842.

Cadogan BL, Scharbach RD, Krause RE, Knowles KR. 2002. Evaluation of tebufenozide carry-over and residual effects on spruce budworm (Lepidoptera: Tortricidae). J. Econ. Entomol. 95: 578-586.

Cônsoli FL, Parra JRP, Hassan SA. 1998. Side-effects of insecticides used in tomato fields on the egg parasitoid *Trichogramma pretiosum* Riley (Hym., Trichogrammatidae), a natural enemy of *Tuta absoluta* (Meyrick) (Lep., Gelechiidae). J. Appl. Ent. 122: 43-47.

Dhadialla TS, Carlson GS, LeDat P. 1998. New insecticides with ecdysteroidal and juvenile hormone activity. Annu. Rev. Entomol. 43: 545–569

Graham LD, Johnson WM, Pawlak-Skrzeck A, Eaton RE, Bliese M, Howell L, Hannan GN, Hill RJ. 2007. Ligand binding by recombinant domains from insect ecdysone receptors. Insect Biochem. Mol. Biol. 37:611-626.

Hahn T, Liess M, Schulz R. 2001. Effects of the hormone mimetic insecticide tebufenozide on *Chironomus riparius* larvae in two different exposure setups. Ecotoxicol. Environ. Saf 49: 171-178.

Kreutzweiser DP, Capell SS, Wainio-Keizer KL, Eichenberg DC. 1994. Toxicity of a new molt-inducing insecticide (RH-5992) to aquatic macroinvertebrates. Ecotoxicol. Environ. Saf. 28: 14–24.

Kreutzweiser DP, Gunn JM, Thompson DG, Pollard HG, Faber MJ. 1998. Zooplankton community responses to a novel forest insecticide, tebufenozide (RH-5992), in littoral lake enclosures. Can. J. Fish. Aquat. Sci. 55: 639–648.

Kropp RK, Pinza MR, Blanton ML. 2004. Draft detailed review paper on aquatic arthropods in life cycle and two- generation toxicity tests. BATTELLE, November, 10 2004, Ohio.

Kwak IS, Lee W. 2005. Mouthpart deformity and developmental retardation. Exposure of *Chironomus plumosus* (Diptera: Chironomidae) to tebufenozide. Bull. Environ. Contam. Toxicol. 75: 859-865

Lezzi M, Bergmann T, Mouillet JF, Henrich VC. 1999. The ecdysone receptor puzzle. Arch Insect Biochem. Physiol. 41: 99-106.

OECD 2004. OECD guideline for the testing of chemicals, sediment-water Chironomid toxicity test using spiked water, OECD 219, adopted 13 April 2004.

OECD 2010. OECD guidelines for the testing of chemicals, sediment-water Chironomid life-cycle toxicity test using spiked water or spiked sediment OECD 233, adopted 22 July 2010.

Oetken M, Bachmann J, Schulte-Oehlmann U, Oehlmann J. 2004. Evidence for endocrine disruption in invertebrates. Int. Rev. Cytol. 236: 1–44.

Pest Management Regulartory Agency 1996. Proposed Regulartory Decision Document: Tebufenozide. PRDD96-01- Health Canada consultation document, Ottawa. www.hc-sc.gc.ca/cps-spc/pest/part/consultations/_prdd96-01/index-eng.php. Accessed 28 April 2012.

Reynolds SE, Seth RK, Kaur JJ, Rao DK. 2004. Effects of larval exposure to sublethal concentrations of the ecdysteroid agonists RH-5849 and tebufenozide (RH-5992) on male reproductive physiology in Spodoptera litura. J. Insect. Physiol. 50: 505-517.

Santillo D, Stringer RL, Johnston PA, Tickner J. 1998. The precautionary principles: protecting against failures of scientific method and risk assessment. Mar. Pollut. Bull. 36: 939-950

Smagghe G, Bylemans D, Medina P, Budia F, Avilla J, Viñuela E. 2004. Tebufenozide distorted codling moth larval growth and reproduction, and controlled field populations. Ann. Appl. Biol. 145:291-298.

Smagghe G, Degheele D. 1997. Comparative toxicity and tolerance for the ecdysteroid mimic tebufenozide in a laboratory and field strain of leafworn. J. Econ. Entomol. 90:278-282.

Soin T, Smagghe T. 2007. Endocrine disruption in aquatic insects: a review. Ecotoxicology 16:83-93.

Song MY, Stark JD, Brown JJ. 1997. Comparative toxicity of four insecticides, including imidacloprid and tebufenozide, to four aquatic species. Environ. Toxicol. Chem. 16:2494–2500.

Staal GB. 1975. Insect growth regulator with juvenile hormone activity. Annu. Rev. Entomol. 20:417-460.

Sun X, Barrett BA, Biddinger DJ. 2000. Fecundity and fertility reductions in adult leafrollers exposed to surfaces treated with the ecdysteroid agonists tebufenozide and methoxyfenozide. Entomol. Exp. Appl. 94:75-83.

Sun X, Song Q, Barrett B. 2003. Effect of ecdysone agonists on vitellogenesis and the expression of EcR and USP in codling moth. Arch. Insect Biochem. Physiol. 52:115-129

Sundaram KMS. 1997. Persistence of tebufenozide in aquaticecosystems under laboratory and field conditions. Pestic. Sci. 51:7–20

Sundaram M, Palli SR, Krell PJ, Sohi SS, Dhadialla TS, Retnakaran A. 1998. Basis for selective action of a synthetic molting hormone agonist, RH-5992 on lepidopteran insects. Insect Biochem. Mol. Biol. 28:693–704.

Süß A, Bischoff G, Mueller CW, Buhr L. 2006. Chemical and biological monitoring of the load of plant protection products and of zoocenoses in ditches of the orchard region "Altes Land". Nachrichtenbl. Deut. Pflanzenschutzd. 58:28-42.

Taenzler V, Bruns E, Dorgerloh M, Pfeifle V, Weltje L. 2007. Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. Ecotoxicology 16:221-230.

Tarrant AM, Behrendt L, Stegeman JJ, Verslycke T. 2011. Ecdysteroid receptor from the American lobster Homarus americanus: EcR/RXR isoform cloning and ligand-binding properties. Gen Comp Endocrinol 173: 346-355.

Tassou KT, Schulz R. 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotoxicol Environ Saf 72: 1058-1062.

Tassou KT, Schulz R. 2011. Two-generation effects of the chitin synthesis inhibitor, teflubenzuron, on the aquatic midge Chironomus riparius. Ecotoxicol. Environ. Saf. 74:1203-1209.

Tunaz H, Uygun N. 2004. Insect growth regulators for insect pest control. Turk. J. Agric. For. 28:377-387.

Wing KD, Slawecki RA, Carlson GR. 1988. RH 5849, a Nonsteroidal Ecdysone Agonist: Effects on Larval Lepidoptera. Science 241:470-472.

Yokota H, Eguchi S, Nakai M 2011. Development of an in vitro binding assay for ecdysone receptor of mysid shrimp (*Americamysis bahia*). Aquat. Toxicol 105: 708-716.

Appendix III

Combined effects of temperature and pyriproxyfen stress in a full life-cycle test with *Chironomus riparius* (Insecta)

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Abstract

Traditional risk assessment guidelines employ acute or chronic toxicity tests for a maximum of one generation of organisms. These tests are usually performed in the laboratory at a constant standard temperature, although in the field organisms may experience different temperatures, which may be a source of additional stress. Climate change-related temperature shifts may have serious impacts on ectotherms' populations that are key components of the aquatic food chains, particularly in combination with the exposure of pollutants affecting their development. Here, a chronic full life-cycle test with *Chironomus riparius* from the first-instar larvae in the parental (P) until emergence in the subsequent F1 generation was conducted at different temperatures (16 and 24°C) testing the effect of the insect growth regulator pyriproxyfen at 1; 3; 10; 30; and 100 µg/L. The emergence ratios were significantly affected by the interaction between temperature, chemical treatment and generation; showing that at lower temperatures, the negative effects of pyriproxyfen exposure were significantly higher in the F1 generation than in the P generation. The development rate showed that the effects of the interactions were significant in the F1 generation, underlying the importance of extended exposure as a useful amendment in the risk assessment of those agrochemicals potentially influencing developmental and reproductive parameters in intact organisms. Moreover, results demonstrated that any difference from the standard temperature of 20°C may result in additional stress leading to the disruption of biological functions in *C. riparius*, highlighting the interaction between different global change-related variables.

Keywords: Full life-cycle test; Interaction effect; Ectotherms; Temperature shift; Organisms' sensibility.

Introduction

Temperature is one of the key environmental factors in the life of ectotherms as all of their life processes depend on the temperature of the habitat (Donker et al. 1998). Due to the ectothermic nature of all aquatic invertebrates, temperature is an important environmental factor controlling their physiological processes (Heugens et al. 2001) in the field. Temperature may influence the metabolic rate or the locomotory and feeding activity of organisms, thus affecting toxicant uptake, elimination and detoxification rates (Cairns et al. 1975; Doneker et al. 1998). Temperature may influence growth at the individual level (Frouz et al. 2002) or cause changes in population abundance (Hall and Burns 2002). Standard toxicity tests in the laboratory are, however, performed at a constant temperature to optimize performance in the control and isolation of the effect of the chemical in question. In the field however, variable and suboptimal temperatures or conditions may be present during the lifespan of organisms (Holmstrup et al. 2010), possibly altering the effects of chemicals tested when compared to laboratory tests performed under optimal and well-controlled conditions.

As temperature influences physiological processes in ectotherms, an interaction between temperature and chemicals can be expected especially when a chemical acts on specific physiological processes (Heugens et al. 2001). However, the lack of knowledge referring to interactions between chemicals and temperature hampers the extrapolation of laboratory toxicity data to ecosystems (Heugens et al. 2003).

Juvenile hormone (JH) plays an important role throughout the entire life-cycle of insects and adjusts many aspects of insects biology such as development and egg production by females (Oehlmann and Schulte-Oehlmann 2003; Kropp et al. 2004). JH is responsible for the growth of the larvae, while impeding metamorphosis. Many of the recent agricultural insecticides have been intended to interfere with JH or ecdsyteroids. Pyriproxyfen, a JH agonistic substance that impedes larvae from developing into the adult stage was chosen for the present experiment.

Established risk assessment guidelines for example those appropriate to agrochemicals propose acute or chronic tests designed for a maximum duration of one generation of test species (OECD 2004). This method does not provide the ability to adequately assess the effects of these agrochemicals on populations (Stark and Banks 2003; Desneux et al. 2007). Especially, for non-targeted freshwater insects, reliable test systems must be proposed or

existing guidelines improved for the assessment of endocrine disruption (OECD 2006). The performance of studies over generations, with the assessment of reproductive parameters, appears to be a suitable attempt to detect and characterize effects of IGRs (OECD 2006).

In order to provide a scientific basis for understanding the interactions between temperature and pesticide exposure during the lifespan of an aquatic insect, we used an experimental design over two generations including *Chironomus riparius* (ectotherm) maintained at 16 and 24°C. The experiment began with first instar larvae at each generation (Tassou and Schulz 2009). Studies investigating the combined effects of temperature and chemical stress have not previously been performed using a two-generation test with aquatic insects. The experimental design is an extension of the established OECD method for testing of chemicals, the sediment-water chironomid life-cycle toxicity test using spiked water, [10] and allows the assessment of life-long exposure effects covering development and reproductive parameters of the P generation and development of the F1 generation.

Materials and Methods

Test species

C. riparius, is a commonly used test organism in aquatic toxicity tests for which several guidelines are available. Egg ropes to start the study were obtained from our in-house culture (maintained at 20°C). This was established in 2006 using egg ropes from the laboratory of BASF SE.

Test compound

Pyriproxyfen (99.7%) was supplied by BASF SE. It is a pyridine based insecticide which is found to be effective against a variety of arthropods and has been described in detail by Tassou and Schulz (2009). Pyriproxyfen imitates physiological properties of JHs by preventing larvae to become adult, thus preventing their ability to reproduce.

Experimental design

Both experiments were conducted during the same season (December 2010 to February 2011). Before conducting the experiments, organisms were simultaneously acclimated at 16 and 24°C for two generations (the generation time at 16°C was longer (~ 35 d) than at 24°C (~ 21 d) due to the faster development of larvae at 24°C) in two climate chambers with a

light/dark cycle of (16h/8h) and relative air humidity of (70±10%). Twenty first instar larvae were exposed to five nominal concentrations (1, 3, 10, 30, 100 µg/L) of pyriproxyfen in a sediment-water system. The test procedure is a standard test design, using eight replicates per treatment and for solvent control under static conditions. The application scenario was via water according to OECD 219. Each test beaker (600 ml glass) comprised of 400 ml M7-medium and 100 g wet formulated non sterilized sediment with a pH of 7.0 ± 0.5 according to OECD 219. Small volumes (40 µl) of pyriproxyfen stock solutions were applied to the water overlying the sediment, 24 hours after addition of the larvae to test beakers for the P generation. For the F1 generation, larvae were transferred directly into freshly prepared and spiked water. The test concentrations were estimated based on the water overlying the sediment, and set up through the dilution of a stock solution made in N, N dimethyformamide (DMF 99.8%). The stock solution was prepared by diluting technical pyriproxyfen in DMF. The feeding of larvae was performed every day with ground food (Tetra Min). During the first 10 d each beaker received 200 µl of a suspension (equal to 500 µg food per larvae per day) made with 3 g ground food and 60 ml M7-Medium. For older larvae, 400 µl suspensions were supplied daily to each test beaker for the rest of the test. The food ratio was then reduced at 50% emergence or if food was observed on the sediment's surface. The emergence and sex ratio of the fully emerged and alive midges were assessed. Emerged adults within a treatment were assigned to two breeding cages (50 cm in all three dimensions), to facilitate swarming, mating and oviposition into a two-liter glass crystallising dish filled with 1000 ml non-aerated M7-medium and 0.3 kg wet formulated sediment. After egg ropes were collected from the crystallising dish they were placed in 12-well microtiter plates containing water from the spiked crystallising dish to assess the fecundity and fertility of the P generation. According to the OECD guideline 233 (OEDC 2010), the fecundity was defined as the number of egg ropes per female and the fertility of an egg rope was assessed within 6 days after it was produced. An egg rope was considered fertile when at least one third of the eggs hatched. To start the F1 generation, six fertile egg ropes of the P generation were selected around test day 19 (the peak of oviposition) from each breeding cage. After hatching, twenty first-instar larvae were allocated randomly to each of the freshly prepared test beakers for the F1 generation. The exposure duration was 28 d for the P generation and longer for the F1 generation as the exposure period commenced at the egg stage.

In addition to the endpoints already established by the OECD guideline 219 (OECD 2004), the present study allowed the assessment of adverse effects on reproduction of the P generation as well as on the development until emergence in the F1 generation. The mean development rate of the midges was defined as the reciprocal of the mean development time and represents the mean larval development which takes place per day (OECD 2010). The mean development rate per vessel ($\bar{x}$) is calculated according to:

$$\bar{x} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$ where

x = mean development rate per vessel

i = index of inspection interval

m = maximum number of inspection intervals

f_i = number of midges emerged in the inspection interval *i;*

n_e = total number of midges emerged at the end of experiment ($= \sum f_i$)

x_i = development rate of midges emerged in interval *i;*

$$x_i = \frac{1}{\left(day_i - \frac{l_i}{2}\right)}$$ where

dayi = inspection day (d since introduction of larvae)

l_i = length of inspection interval i (days, usually 1 d) (OEDC 2010).

Chemical analysis

For the chemical analysis, samples were prepared using the solid phase extraction procedure. Water samples (400 ml) taken from additional test vessels at the beginning and the end of the test for the P generation were buffered at pH 3 and passed through a Chromabond SPE C18 cartridge after conditioning of the cartridge with acetonitrile. The pyriproxyfen present in the sample was selectively adsorbed onto the cartridge material, which was then immediately frozen at -20°C until analysis. Before analysis, pyriproxyfen was eluted with acetonitrile from the cartridge. All samples were then analysed by an Exactive LC-MS instrument equipped

with a CombiPal autosampler (CTC Analytics), an Accela pump, and Surveyor LC pump (ThermoFisher Scientific). Purified water containing 0.1% formic acid and 4 mmol ammonium formate (both Sigma Aldrich,puriss. p.a. grade) (solvent A) and acetonitrile (hypergrade, Merck) containing 0.1% formic acid and 4 mmol ammonium formate (solvent B) served as eluents. 500 µl of the aqueous sample were injected on the EQuan system equipped with a Hypersil Gold aQ column (20 × 2.1 mm; particle size 12 µm) for concentration with a flow rate of 2 ml/min of 98% solvent B and 2% solvent A.

The sample was transferred on-line to the analytical column; a Hypersil Gold C18 column purchased from Thermo Fisher Scientific (50 × 2.1 mm, particle size 1.9 µm) with a flow rate of 200 µl/min. The gradient began with 95% A and 5% B for 2 minutes, increased to 100% B in 2 minutes and maintained for 4 min. The analytical column was conditioned for 2.5 min with 95% A and 5% B. The ionization of analytes was assured using an Ion Max API Source with an ESI probe, operated at room temperature with a spray voltage of 3.6 kV. Nitrogen was used as sheet gas and auxiliary gas with flow rates of 20 ml/min and 5 ml/min, respectively. The ions were detected in the scan range of m/z 100-2000 in the positive mode. The compound was identified using the accurate mass of the $[M+H]^+$ with deviations always smaller than 5 mg/L between theoretical and measured mass. For quantification the $[M+H]^+$ together with an external calibration were used. The analytical results are reported in Table 1 showing small deviations between temperatures for the 3 and 10 µg/L treatments, but for the 100 treatment a 25% lower concentration was measured. However, these two treatments did not show any emergence of midges in the P generation.

Table 1: Nominal and mean measured concentrations (±SD; n = 2) of pyriproxyfen for the P generation at16 and 24°C measured in the overlying water at day 0 and day 28.

Temperature	Nominal concentration (µg/L)	Measured Concentration (µg/L)	
		Day 0	Day 28
16°C	Solvent control	0	< LOD
	3	2.8±0.0003	< LOD
	10	8.8±0.0004	< LOD
	100	72±0.004	< LOD
24°C	Solvent control	0	< LOD
	3	2.7±0.0001	< LOD
	10	9.1±0	< LOD
	100	65±0.006	< LOD

LOD: limit of detection (LOD = 0.04 µg/L); LOQ: limit of quantification (LOQ = 0.15 µg/L)

Data analysis

The emergence ratio data of the P and F1 generations were arcsin-sqrt transformed for a normal distribution and to improve variance homogeneity. Dunnett's test was used for this endpoint to compare the treatments with the corresponding solvent control at each temperature regime. The mean difference was significant at the 0.05 level. Additionally, factorial analysis of variance was performed to evaluate potential interaction effect of temperature (16 vs. 24°C), chemical exposure (control vs. treatments) and generation (P vs. F1) on the life history parameters of *C. riparius*. For the fecundity and fertility, Dunnett's test was used to compare the treatments with the corresponding solvent control at each temperature regime. A dose-response relationship and toxicity data such as the concentration

causing 50% impairment (LC_{50} or EC_{50}) on life history parameters of *C. riparius* were calculated with the software program BioStat 2009.

Results

Emergence ratio

The total number of emerged midges for the P or F1 generation in controls did not differ among temperatures. At 16°C, a dose-related effect on emergence ratio was observed at a pyriproxyfen concentration ≥ 10 µg/L in the P generation, while in the F1 generation a significant effect was observed already at 1 µg/L compared to the corresponding solvent control (Fig. 1). Median effect concentrations (EC_{50}) values of 6.1 µg/L (95% CI, 3.2-11.3) and 1.2 µg/L (95% CI, 0.9-1.5) were estimated for the P and F1 generation, respectively.

At 24°C, a decrease in the emergence ratio of the P generation was observed at 10 µg/L, which was also the lowest treatment value showing a substantial deviation from the control, i.e, an emergence ratio reduced almost to zero. In the F1 generation no decrease in emergence was observed for the concentration ranging up to 3µg/L. This concentration of 3µg/L was also the highest concentration tested in the F1 generation due to the high mortality at higher concentrations in the P generation (Fig. 1). The estimated EC50 for the P generation was 4.1µg/L (95% CI, 2.2-7.5). For the F1 generation, it was judged not necessary to calculate the EC_{50}-value since the emergence ratios in all treatments ranging up to 3µg/L were over 86%.

Effects on the emergence ratio in the F1 generation were more pronounced at 16°C than at 24°C (Fig.1). We observed an earlier emergence of midges at 24°C than at 16°C. A significant combined effect of temperature and pyriproxyfen ($p < 0.001$) was found for the emergence ratio in the P generation as well as in the F1 generation. A significant three factorial interaction effect of temperature × chemical × generation ($p < 0.001$) was determined for the emergence ratio of the midges (Table 2). This shows that at the lower temperature, the negative effects of pyriproxyfen in the F1 generation were significantly higher than in the P generation.

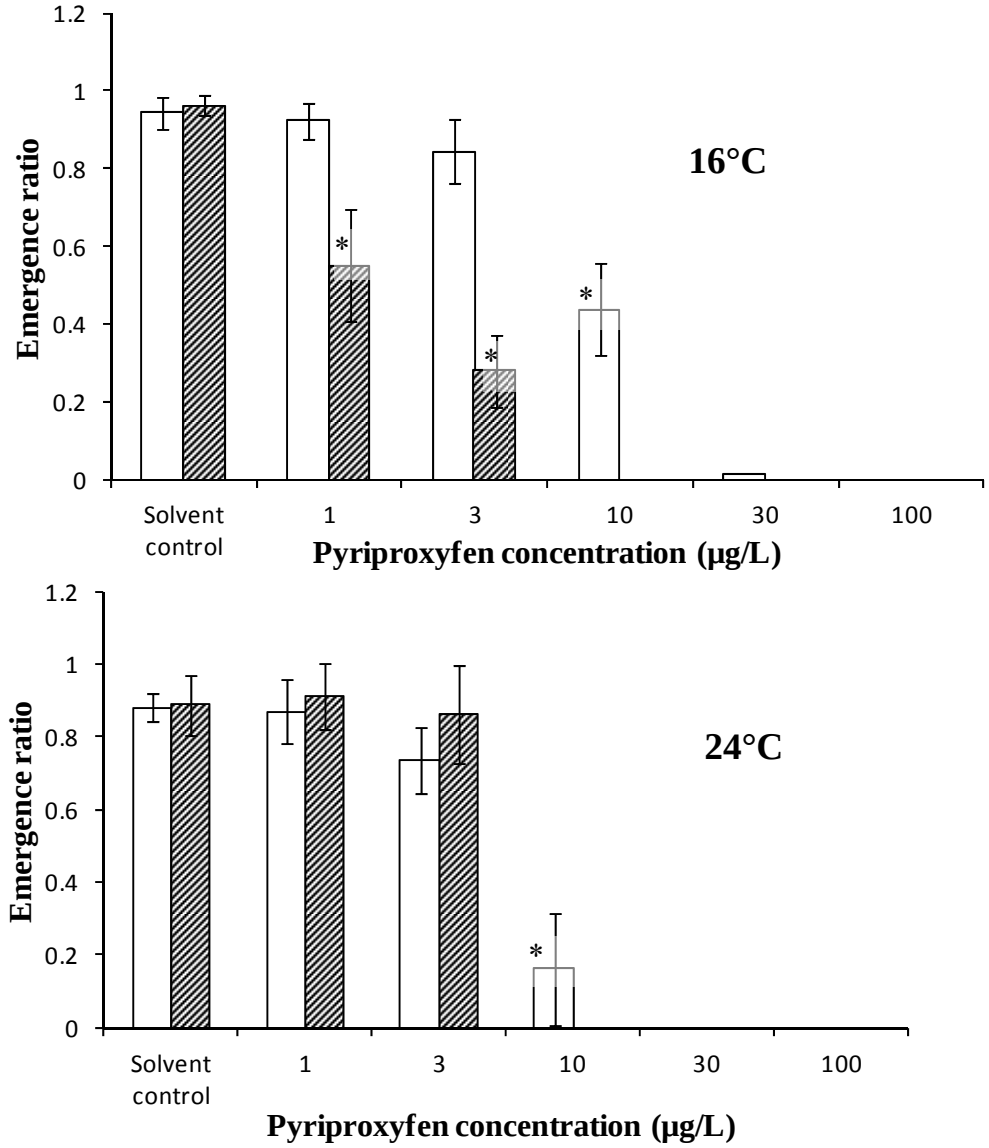

Fig. 1 Emergence ratio (±SD; n= 8) of *C. riparius* in the P generation (white bars) and F1 generation (hatched bars) at different temperatures during static exposure to pyriproxyfen. Asterisks denotes for each temperature regime and in each generation significant differences (Dunnett's test) from the corresponding solvent control.

Table 2: Factorial analysis of variance of the effect of temperature (16 vs. 24°C), chemical exposure (control vs. pyriproxyfen treatments) and generation (P vs. F1) on life parameters of *C. riparius*.

Endpoint	Source	df	MS	F	P
Emergence ratio	Temperature	1	0.278	8.957	0.003
	Chemical	5	6.153	197.934	< 0.001
	Generation	1	0.261	8.387	0.004
	Temperature × Chemical	5	0.207	6.665	< 0.001
	Temperature × Generation	1	1.261	40.547	< 0.001
	Chemical × Generation	2	0.178	5.721	0.004
	Temperature × Chemical× Generation	2	0.414	13.305	< 0.001
Development rate	Temperature	1	0.021	2.527E3	< 0.001
	Chemical	3	0.000	19.313	< 0.001
	Generation	1	0.000	57.322	< 0.001
	Temperature × Chemical	3	2.668E-5	3.155	0.028
	Temperature ×Generation	1	2.574E-6	0.304	0.582
	Chemical × Generation	2	1.628E-6	0.193	0.825
	Temperature × Chemical× Generation	2	1.872E-5	2.213	0.115

df = degrees of freedom; MS = mean square; F = likelihood ratio; p = probability

Development rate

The effect of temperature alone on the mean development rate of *C. riparius* can be determined by comparing the development rates in the controls. As hypothesised , the development time decreased as temperature increased (Fig. 2). At both 16 and 24°C, a lower development rate of control midges was observed in the F1 generation when compared to the

P generation (Fig. 2) showing behaviour of organisms supposedly still not acclimated fully to the used temperatures.

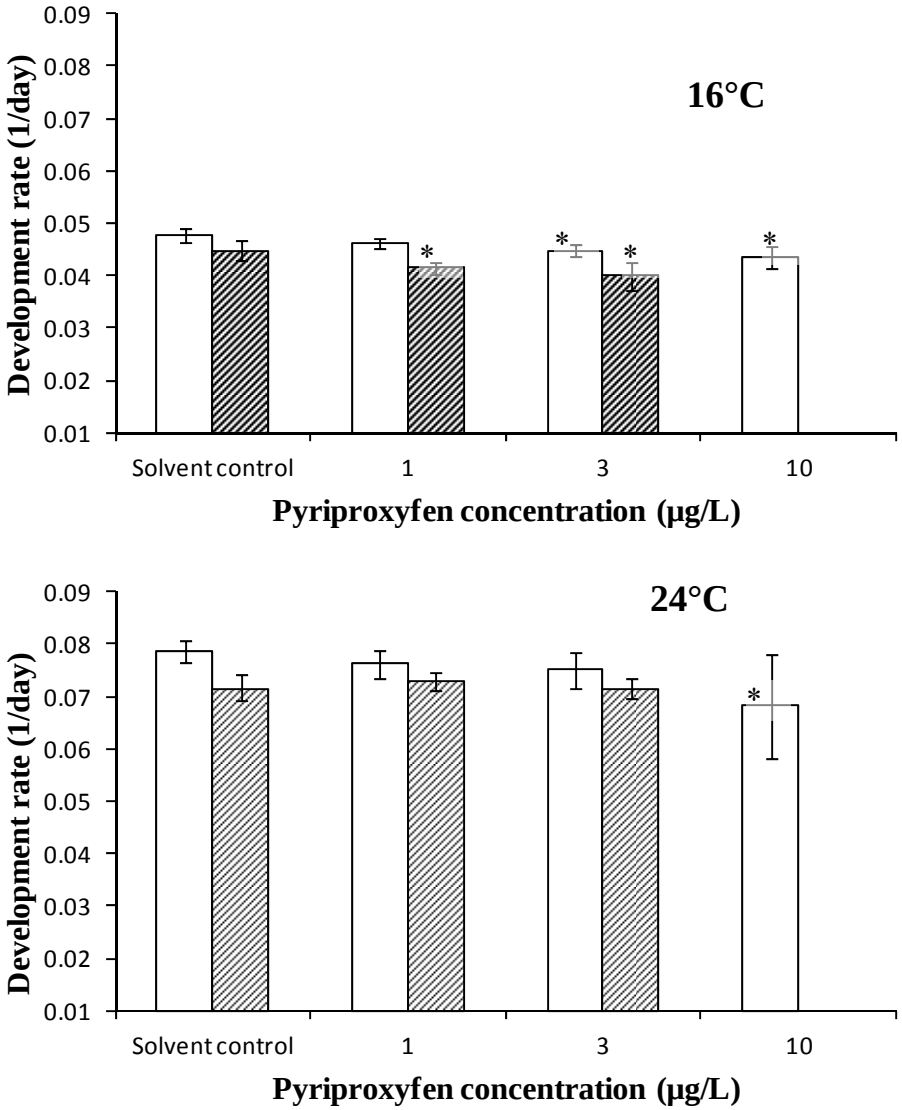

Fig. 2 Mean development rate (±SD; n= 8) of *C. riparius* at different temperatures in the P generation (white bars) and the F1 generation (hatched bars) with pyriproxyfen exposure. Asterisks denotes for each temperature regime and in each generation significant differences (Dunnett's test) from the corresponding solvent control.

Responses in mean development rate in treatments varied among the different temperature regimes. At 16°C, a significant reduction of the mean development rate in exposed midges was observed at 3 µg/L in the P generation compared to solvent controls. In the F1 generation, a significant effect on the mean development rate of midges was observed already at 1 µg/L

(Fig.2). Effective concentrations that cause ten percent effect (EC_{10}) on development of midges were calculated and gave values of 2.98 µg/L (95% CI, 0.36-3.72) and 1.69 µg/L (95% CI, 0.78-2.60) for the P and F1 generation, respectively.

At 24°C, the mean development rate of exposed midges in the P generation differed from solvent control only at 10 µg/L. In the F1 generation, no effects on development rate were observed for the concentration range of 1 µg/L - 3 µg/L. In treatments with the highest concentrations of 10 µg/L - 100 µg/L, it was not possible to conduct the F1 generation since no fertile egg ropes were obtained from the P generation. EC_{10} values of 2.77 µg/L (95% CI, 2.12-3.42) and 2.81µg/L (95% CI, 1.79-3.52) were estimated for the P and F1 generation, respectively. No significant combined effect of temperature and chemical on the mean development rate (p = 0.158) was found in the P generation, while a significant effect (p = 0.003) of these combined factors was present in the F1 generation (Table 3). However, no significant interaction effects of temperature, chemical treatment and generation (p = 0.115) were found on the mean development rate of the midges, though each of the stressors temperature, chemical and generation alone indicated a significant effect on development rate (Table 2). Significant interaction effects on development rate of midges were also observed in the temperature × chemical (p = 0.028) but not for temperature × generation and chemical × generation combined (Table 2).

Table 3: Factorial analysis of variance of the effect of temperature (16 vs. 24°C) and chemical exposure (control vs. pyriproxyfen treatments) on the development rate of *C. riparius* for both parental (P) and filial (F1) generation.

	Source	**df**	**MS**	**F**	**p**
Development rate (P)	Temperature	1	0.013	1.063E3	< 0.001
	Chemical	3	0.000	12.106	< 0.001
	Temperature × Chemical	3	2.156E-5	1.804	0.158
Development rate (F1)	Temperature	1	0.011	2.643E3	< 0.001
	Chemical	2	2.952E-5	7.304	0.002
	Temperature × Chemical	2	2.640E-5	6.531	0.003

df = degrees of freedom; MS = mean square; F = likelihood ratio; p = probability

Sex ratio

The sex ratio expressed as the male fraction, exhibited no adverse effect in both P and F1 generation at all temperature levels. The mean male fraction in controls of both generations fulfilled the validity criteria for sex ratio according to OECD guideline 233 (OECD 2010) (sex ratio between 0.4 and 0.6) at all temperature regimes. In the exposed groups, the mean male fraction was also within this range, indicating no adverse effect of pyriproxyfen on the sex ratio of *C. riparius*.

Fecundity

The number of egg ropes produced per female was assessed for the P generation at each temperature. In the controls, no difference in the number of egg ropes per female was observed at 24°C (0.77) and 16°C (0.81). For the treatments at both 16 and 24°C, a significant effect on fecundity was observed already at 3 µg/L compared to the corresponding control (Fig. 3). EC_{50} values of 3.1 µg/L (95% CI, 1.4 - 7.1) at 16°C and 2.7 µg/L (95% CI, 1.8 – 6.5) at 24°C were estimated.

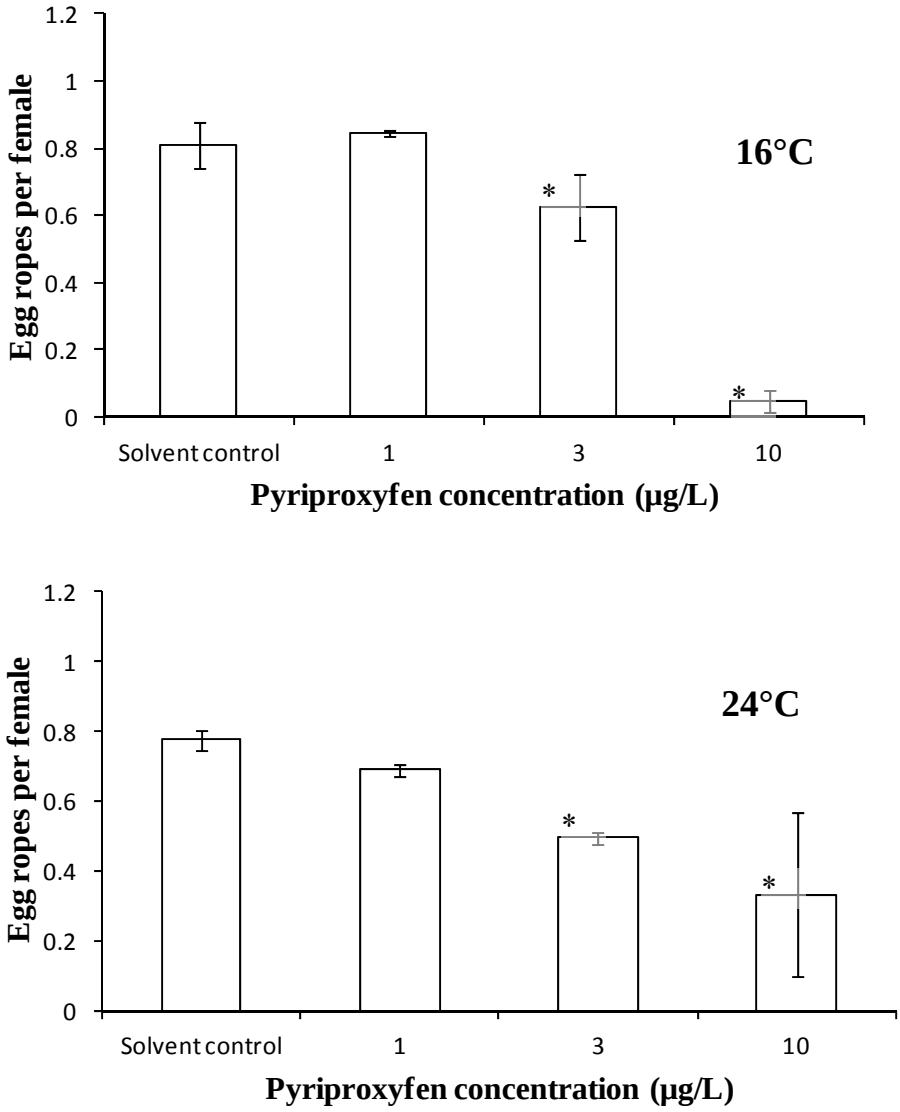

Fig. 3 Egg ropes per female of *C. riparius* in the P generation at 16 and 24°C after pyriproxyfen exposure under static conditions. Asterisks denote at each temperature regime, significant differences compared to the solvent control.

Fertility

High fertility of egg ropes was observed for controls at all considered temperatures, with over 84% of the laid egg ropes being fertile at each temperature. In the treated groups, the average fertility was above 69% excluding the treatment of 10 µg/L at 16°C in which, no fertile egg rope was observed.

Discussion

The present full life-cycle test indicated that exposure to sub-lethal environmentally relevant concentrations of pyriproxyfen at different temperatures affect developmental and reproductive processes of *C. riparius*. It demonstrated that any shift in temperature from the standard temperature at which toxicity tests are performed in the laboratory (usually 20°C), could result in additional stress leading to disruption of biological functions in the organisms. A significant interaction effect was observed between temperature and pyriproxyfen exposure on the emergence ratio ($p < 0.001$) for both P and F1 generation. Specifically the emergence ratio of *C. riparius* was affected by the significant interaction between temperature, chemical exposure and generation ($p < 0.001$; Table 2). This means that both at 16 and 24°C midges were experiencing suboptimal conditions. This could be a source of additional stress altering effects of pesticides in comparison to laboratory tests usually performed at 20°C that might represent a best case scenario. Vinebrooke et al. (2004) suggested that an understanding of the interactive effects of multiple stressors (such as pollutants and temperature) and their mechanisms is critical for predicting the tolerance limits, survival and productivity of ectotherm populations and for modelling the effects of global climate change on aquatic ecosystems.

Temperature stress effects on organisms have been largely investigated; however studies on interactions of toxic compounds and temperature are relatively new and not completely understood (Harwood et al. 2009; Holmstrup et al. 2010). Weston et al. (2009) reported that pyrethroid toxicity to the aquatic amphipod *Hyalella azteca* was highly temperature dependent. Holmes et al. (2008) also used the characteristic of greater toxicity of pyrethroids at lower temperatures as a diagnostic tool to investigate the incidence of pyrethroid associated toxicity of ambient urban sediments to *H. azteca*. Although some reviews on the acute or chronic (for maximal one generation) interactions between temperature and chemicals exist (Cairns et al. 1975; Mayer and Ellersieck 1988; Heugens et al. 2001; Holmstrup et al. 2010), the present data with *C. riparius* are to our knowledge, the first results showing a significant interaction between temperature, chemical exposure and generation in a full life-cycle test (two-generation study). The results therefore underline the importance of considering effects of combined factors over multiple generations of species in the risk assessment. This aspect is particularly important since it may reflect the situation organisms usually experience during their lifetime in their natural habitats.

For a 4 degree temperature deviation from 20°C, EC_{50} values for the emergence ratio indicated that midges were 1.49 times more sensitive to pyriproxyfen at 24°C in the P generation than at 16°C. These temperature-related toxicity effects were similar to those reported by Mayer and Ellersieck (1988) who reviewed the effects of temperature on the acute toxicity of aquatic organisms and indicated that most organic chemicals exhibit a two-to fourfold change in toxicity for each 10°C change in water temperature. Heugens et al. (2001) also claimed that organisms living under conditions close to their environmental tolerance limits appeared to be more vulnerable to additional chemical stress. However, it is known that, within a single species, several ecotypes commonly occur after a long adaptation (six generations for *Drosophila melanogaster* reported by Bakker et al. 2010) to specific environmental conditions (e.g. temperature regimes) in the populations. No comparison has been made in the F1 generation at both temperatures as the emergence ratio at 24°C did not show any effect in the F1 generation. A comparison of effects on the emergence ratio of the midges at 16°C indicated however, a five times more sensitivity to pyriproxyfen in the F1 generation than did the P generation.

As temperature increased, metabolism increased, as did the chemical uptake and possible biotransformation and elimination of the chemical (Lydy et al. 1999). This is most likely the reason for the slight decrease in toxicity observed in the F1 generation at 24°C in the concentration range of1 to 3 µg/L, though effects were observed at 16°C. A decrease in toxicity corresponding to an increase in temperature was also reported for some organochlorine and most pyrethroid insecticides (Ferrando et al. 1987; Harwood et al. 2009; Howe et al. 1994). Harwood et al. (2009) showed greater sensitivity of *Chironomus dilutus* to pyrethroids at lower temperatures while chlorpyrifos (a metabolically activated pesticide) showed the opposite effect of decreased toxicity with decreasing temperatures. The authors suggested that the influence of temperature on biotransformation of pyrethroids and chlorpyriphos could explain the overall trends of temperature on toxicity of the aforementioned insecticides. Cairns et al. (1975) suggested that an increase in detoxification mechanisms and excretory processes at high temperature levels may counteract the effect of the temperature. Vogt et al. (2007) in their investigation of temperature effect on genetic diversity in *C. riparius* populations, found no temperature effect on genetic diversity in the F1 generation at 23°C for a genetic diverse population, while Imasheva et al. (1997) suggested

that the effects of temperature stress at population level may be chronic within the genetic architecture of the population.

The observed increase in the development rate of *C. riparius* with increasing temperature supported observations by Oetken et al. (2009), who reported an obvious growth advantage in the control compared to tributyltin-treatment over a temperature range of 17-23°C. Moreover, the differences observed in development rates of controls between generations at both 16 and 24°C, could be due to organisms not completely adapted to the new temperatures (Heugens et al. 2001). A previous study in our laboratory with organisms from the culture maintained at 20°C, showed no difference in controls' development rate between generations (Tassou and Schulz 2009). The precluding two generation acclimation of the midges at 16and 24°C may not have been long enough to select individuals with another temperature optimum. No M7 water control was conducted in addition to the solvent control in the present study due to the high number (eight) of replicates of each of the five chosen concentrations. In addition, the solvent (DMF) did not show any significant effect on midges in a previous study conducted with the same compound at 20°C in our laboratory (Tassou and Schulz 2009). These results showed that adaptation time might extent to several generations during which, organisms especially ectotherms might be more vulnerable to additional chemical stress. However, this acclimation time was longer than the two weeks used by Donker et al. (1998) with the isopod *Porcellio scaber*. This should have been long enough to assure the synchronization of adult emergence. Geister and Fischer (2007) also reared the butterfly *Bicyclus anynana* for two generations at 27°C prior to experiments, in which they tested the beneficial acclimation hypothesis. This observation confirmed the assertion that temperature is an important factor having a significant impact on the rate of most physiological processes in ectotherms (Heugens et al. 2003). Some authors working on the growth pattern of chironomidae reported, that the development rates increased corresponding temperature until a certain limit was reached (Frouz et al. 2002; Péry and Garric 2006; Stevens 1998). All these studies referenced here were conducted within a temperature range of 12.5°C to 37.5°C that includes the chosen temperature regimes (16 and 24°C) in the current study. An increase in the population growth rate of *C. riparius* with increasing temperature was reported, possibly due to a direct influence of temperature on metabolism (Donker et al. 1998). It could also be due to a shortening of the intermoult period at increasing temperature (Widianarko et al. 1994).

The more sensitivity of the development rate in the F1 generation at 16°C was in accordance with previous studies in our laboratory and emphasized the importance of conducting multiple generation studies to assess additional information as a useful amendment for the risk assessment for persistent pesticides which may interfere with the endocrine system of organisms.

The no significant interaction of temperature × pyriproxyfen exposure × generation (p = 0.115; Table 2) found on the development rate of midges, suggested that the emergence ratio was more sensitive than the development rate in the present study. These results showed that different endpoints may be individually sensitive to the same interaction, underlining the importance of considering multiple factors in assessment of risk brought by exposure to toxic chemicals in natural conditions as also suggested by Laskowski et al. (2010).

When considering only controls, no difference was observed between the number of egg ropes per female for the considered temperature regimes (0.81 at 16°C and 0.77 at 24°C). No effect on the number of produced egg-masses per female was observed by Vogt et al. (2007) in their study on the interaction between genetic diversity and temperature stress on life–cycle parameters and genetic variability in *C. riparius* populations.

In the pyriproxyfen treatments, a decrease in fecundity was observed within the same temperature regime. A factor of ≥ 6 between values were to be found if one have to compare EC_{50} values at the optimum temperature of 20°C in our laboratory, with those at 16°C and 24°C. This underlines a combined effect of temperature and pyriproxyfen on fecundity at temperatures different from this optimum. A decrease in the number of egg clutches by females of *C. riparius* between the 20 and 23°C tributyltin treatment was reported (Ferrando et al. 1987). Jacobson et al. (2008) also reported that elevated temperature and the fungicide fenarimol interacted synergistically and reduced the fecundity of the amphipod *Monoporeia affinis*. Moreover, a decrease in fecundity of *Daphnia magna* at 20°C due to pyriproxyfen exposure and a dose related adverse effects of some juvenile hormone analogues was reported by Tatarazako et al. (2003).

Conclusion

Our results indicated that there were possible interactions between a pesticide and temperature, which may alter biological functions of organisms. The dose-related decrease in fecundity observed in the pyriproxyfen treatments at each temperature regime, suggested that temperature changes could impede the populations of *C. riparius*. Moreover, the present test design may contribute to our understanding of how responses of experimental populations vary across temperature gradients and different levels of chemical stressors.

Knowing how exposed populations may vary in their response to combined temperature and pesticide stress during a complete life cycle is important in predicting impacts that will occur in the field. Therefore, long-term studies designed to consider the chemical-environment interactions addressing the ecological complexity, may provide important information relevant in the process of ecological risk assessment of chemicals, particularly those acting as endocrine disrupters.

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References

Bakker J, van Rijswijk MEC, Wessing FJ, Bijlsma R. 2010. Consequences of fragmentation for the ability to adapt to novel environments in experimental *Drosophila* metapopulations. Conserv. Genet. 11:435-448.

BioStat. 2009. User friendly biology and medicine oriented software, Version 2, Type: Application. Publisher: AnalystSoft Inc.

Cairns J, Heath AG, Paker BC. 1975. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia 47:135-171.

Desneux N, Decourtye A, Delpuech JM. 2007. The sublethal effects of pesticides on beneficial arthropods. Annu. Rev. Entomol. 52:81-106.

Donker MH, Abdel-Lateif HM, Khalil MA, Bayoumi BM, Van Straalen NM. 1998. Temperature, physiological time, and zinc toxicity in the isopod *Porcellio scaber*. Environ. Toxicol. Chem. 17:1558-1563.

Ferrando MD, Andreu-Moliner E, Almar MM, Cebrian C, Nunez A. 1987. Acute toxicity of organochlorine pesticides to the European eel, *Anguilla anguilla*: the dependency on exposure time and temperature. Bull. Environ. Contam. Toxicol. 39:365-369.

Frouz J, Ali R, Lobinske RJ. 2002. Influence of temperature on development rate, wing length, and larval head capsule size of pestiferous midge *Chironomus crassicaudatus* (Diptera: Chironomidae). J. Econ. Entomol. 95:699-705.

Geister TL, Fisher K. 2007. Testing the beneficial acclimation hypothesis: temperature effects on mating success in a butterfly. Behav. Ecol. 18:658-664.

Hall CJ, Burns CW. 2002. Mortality and growth responses of *Daphnia carinata* to increases in temperature and salinity. Freshw. Ecol. 17, 169-170.

Harwood AD, You J, Lydy MJ. 2009. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. Environ. Toxicol. Chem. 28:1051-1058.

Heugens EHW, Hendriks AJ, Dekker T, van Straalen MM, Admiraal W. 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for the use in risk assessment. Crit. Rev. Toxicol. 31:247-284.

Heugens EHW, Jager T, Creyghton R, Kraak MHS, Hendriks AJ, van Straalen NM, Admiraal W. 2003. Temperature-dependent effects of cadmium on *Daphnia magna*: accumulation versus sensitivity. Environ. Sci. Technol. 37:2145-2151.

Holmes RW, Anderson BS, Phillips BM, Hunt JW, Crane DB, Mekebri A, Connor V. 2008. Statewide investigation of the role of pyrethroid pesticides in sediment toxicity in California's urban waterways. Environ. Sci. Technol. 42:7003-7009.

Holmstrup M, BindesbØl AM, Oostingh GJ, Duschl A, Scheil V, Köhler HR, et al. 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Sci. Total. Environ. 408:3746-3762.

Howe GE, Marking LL, Bills TD, Rach JJ, Mayer FL. 1994. Effects of water temperature and pH on toxicity of terbufos, trichlorfon, 4-nitrophenol and 2,4-dinitrophenol to the ampipod *Gammarus pseudolimnaeus* and rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 13:51-66.

Imasheva AG, Loeschcke V, Zhivotovsky LA, Lazebny OE. 1997. Effects of extreme temperature on phenotypic variation and developmental stability in *Drosophila melanogaster* and *Drosophila buzzatii*. Biol. J. Linn. Soc. 61:117-126.

Jacobson T, Prevodnik A, Sundelin B. 2008. Combined effects of temperature and a pesticide on the Baltic amphipod *Monoporeia affinis*. Aquat. Biol. 1:269-276.

Kropp RK, Pinza MR, Blanton ML. 2004. Draft detailed review paper on aquatic arthropods in life cycle and two- generation toxicity tests, Battelle, Ohio, November, 10 2004.

Laskowski R, Bednarska AJ, Kramarz PE, Loureiro S, Scheil V, Kudlek J, et al. 2010. Interactions between toxic chemicals and natural environmental factors- A meta-analysis and case studies. Sci. Total. Environ. 408:3763-3774.

Lydy MJ, Belden JB, Ternes MA. 1999. Effect of temperature on the toxicity of M-parathion, chlorpyrifos, and pentachlorobenzene to *Chironomus tentans*. Arch. Environ. Contam. Toxicol. 37:542-547.

Mayer FL, Ellersieck MR. 1988. Experiences with single-species tests for acute toxic effects on freshwater animals. Ambio 17:357-367.

Oehlmann J, Schulte-Oehlmann U. 2003. Endocrine disruption in invertebrates. Pure Appl. Chem. 75:2207-2218.

Oetken M, Jagodzinski LS, Vogt C, Jochum A, Oehlmann J. 2009. Combined effects of chemical and temperature stress on *Chironomus riparius* populations with differing genetic variability. J. Environ. Sci. Health Pt. A 44:955-962.

Organisation for Economic Co-operation and Development. 2004. Guideline for the testing of chemicals, sediment-water Chironomid toxicity test using spiked water, OECD 219, adopted 13 April 2004.

Organisation for Economic Co-operation and Development. 2006. Detailed review paper on aquatic arthropods in life cycle toxicity tests with an emphasis on developmental, reproductive and endocrine disruptive effects. OECD document, No 55, Paris, France.

Organisation for Economic Co-operation and Development. 2010. Guidelines for the testing of chemicals, sediment-water Chironomid life –cycle test using spiked water or spiked sediment. OECD 233, adopted 22 July 2010.

Péry ARR, Garric J. 2006. Modelling effects of temperature and feeding level on the life cycle of the midge *Chironomus riparius*: Hydrobiologia 553:59-66.

Stark JD, Banks JE. 2003. Population-level effects of pesticides and other toxicants on arthropods. Annu. Rev. Entomol. 48:505-519.

Stevens MM. 1998. Development and survival of *Chironomus tepperi* Skuse (Diptera: Chironomidae) at a range of constant temperatures. Aquat. Insect 20:181-188.

Tassou KT, Schulz R. 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotoxicol. Environ. Saf. 72:1058-1062.

Tatarazako N, Oda S, Watanabe H, Morita M, Iguchi T. 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53:827-833.

Vinebrooke RD, Cottingham KL, Norberg J, Scheffer M, Dodson SI, Maberly C, et al. 2004. Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. Oikos 104:451-457.

Vogt C, Pupp A, Nowak C, Jagodzinski LS, Baumann J, Jost D, et al. 2007. Interaction between genetic diversity and temperature stress on life-cycle parameters and genetic variability of *Chironomus riparius* populations. Climate Res. 33:207-124.

Weston DP, You J, Harwood AD, Lydy MJ. 2009. Whole sediment toxicity identification evaluation tools for pyrethroid insecticides: III. Temperature manipulation. Environ. Toxicol. Chem. 28:173-180.

Widianarko B, Donker MH, Van Straalen NM. 1994. Arrhenius activation energy of three *Porcello scaber* (Isopoda) populations: An evaluation of cadmium effects. In Widianarko B, Vink K, Van Straalen NM, eds, Environmental Toxicology in South East Asia. Vrije Universiteit, University Press, Amsterdam, The Netherlands, pp 199-208.

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Jan.-March 2008	Participation in a limited non-GLP ring test with *C. riparius* to extension of OECD Guidelines 218/219 With the participation of BASF, BAYER Crop Sciences and SYNGETA
Dec. 2007	Diploma thesis at the University of Koblenz-Landau: „Multigenerational study with *Chironomus riparius* to assess endocrine disrupting chemicals"
Since April 2006	Successful breeding of the non-biting midge *Chironomus riparius* in the laboratory Successful establishment of a full life-cycle test with *C. riparius*
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Oct. 2005- Dec. 2007	Environmental Sciences at the University of Koblenz-Landau, Campus Landau, Germany, with emphasis on aquatic ecotoxicology
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Tassou KT, Schulz R. 2011. Two-generation effects of the chitin synthesis inhibitor, teflubenzuron, on the aquatic midge *Chironomus riparius*. Ecotoxicol. Environ. Saf. 74:1203-1209.

Tassou KT, Schulz R. 2012. Combined effects of temperature and pyriproxyfen stress in a full life-cycle test with *Chironomus riparius* (Insecta). Environ. Toxicol. Chem. 31:2384-2390.

Tassou KT, Schulz R. 2012 Low field-relevant tebufenozide concentrations affect reproduction in *Chironomus riparius* (Diptera: Chironomidae) in a long-term toxicity test. Environ. Sci. Pollut. Res. Accepted: 7 November. DOI 10.1007/s11356-012-1311-4.

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