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Hazard identification of imidacloprid to aquatic environment

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ABSTRACT

The use of a very effective insecticide against sucking pests, neonicotinoid imidacloprid, has been increasing extensively. For this reason elevated concentrations are expected in aquatic environment. Despite this fact, there is still a lack of data available on its possible risk for the environment. In this study, the potential hazards of imidacloprid and its commercial product Confidor SL 200 to aquatic environment were identified by the acute and chronic toxicity assessment using bacteria *Vibrio fischeri*, algae *Desmodesmus subspicatus*, crustacean *Daphnia magna*, fish *Danio rerio* and the ready biodegradability determination. We found out, that imidacloprid was not highly toxic to tested organisms in comparison to some other environmental pollutants tested in the same experimental set-up. Among the organisms tested, water flea *D. magna* proved to be the most sensitive species after a short-term (48 h EC50 = 56.6 mg L^{-1}) and long-term exposure (21 d NOEC = 1.25 mg L^{-1}). On the contrary, the intensified toxicity of Confidor SL 200 in comparison to analytical grade imidacloprid was observed in the case of algae and slight increase of its toxicity was detected testing daphnids and fish. The activities of cholinesterase, catalase and glutathione S-transferase of daphnids were not early biomarkers of exposure to imidacloprid and its commercial product. Imidacloprid was found persistent in water samples and not readily biodegradable in aquatic environment. Due to increased future predicted use of commercial products containing imidacloprid and the findings of this work, we recommend additional toxicity and biodegradability studies of other commercial products with imidacloprid as an active constituent.

1. Introduction

Worldwide production and application of pesticides have increased progressively during the last two decades. It is important to know that only a small portion of applied pesticide in the field reaches the final biological target. A great part of applied pesticide is released into the environment, where it can provoke problems, such as toxicity to non-target organisms and accumulation. Polluted soil, surface and ground waters involve risk to the environment and also to human health due to possible direct or indirect exposures. For this reason there is a need to monitor and assess possible adverse effects of applied pesticides on ecosystems (Tomlin, 1997; Wamhoff and Schneider, 1999; Nemeth-Konda et al., 2002).

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine], a new promising insecticide, has been commercially introduced to the market in 1991 by Bayer AG and Nihon Tokushu Noyaku Seizo KK and has been increasingly used ever since. It is a worldwide used insecticide, used mainly to control sucking insects on crops, (e.g. aphids, leafhoppers, thrips, whiteflies, termites) (Tomlin, 1997; Tomizawa and Casida, 2005) and parasites (e.g. fleas) of dogs and cats (Dryden et al., 2000). It is a systemic insecticide used for seed treatment, soil and foliar applications. Imidacloprid belongs to the group of nicotine-related insecticides referred to as neonicotinoids, which act as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) (Matsuda et al., 2001) resulting in the impairment of normal nerve function. It is now considered a possible replacement for the insecticides, which are in the process of phased revocation (US EPA, 2004).

Data on the environmental fate of imidacloprid are rather inconsistent. Some authors consider imidacloprid as relatively immobile in soil and do not expect its leaching to groundwater (Mullins, 1993; Tomlin, 1997; Krohn and Hellpointner, 2002), while some studies indicate the opposite (Felsot et al., 1998; Gonzales-Pradas et al., 1999; Armbrust and Peeler, 2002; Gupta et al., 2002). Literature data reported that in aqueous samples imidacloprid is quite stable to hydrolysis at environmentally relevant pH values (Yoshida, 1989) but it undergoes photolytic degradation rapidly (Hellpointner, 1989; Krohn and Hellpointner, 2002).

Although imidacloprid is not intended for use in water, it may pass into water bodies by spray drift or by run-off after application. In comparison to other widely used insecticides, only few toxicity studies have been performed on the effects of imidacloprid on aquatic organisms despite its increasing use (Jemec et al., 2007).

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It is therefore important to assess the concentrations at which these chemicals are toxic to aquatic organisms. There is also a lack of data on the environmental fate of imidacloprid in the aquatic ecosystems, e.g. biodegradation, bioaccumulation. Furthermore, no attention was paid to the effects of commercial formulations of imidacloprid, e.g. Confidor SL 200, Gaucho, Admire, Provado, which usually contain other toxic ingredients, such as solvents. Namely, possible interactions between the pesticide and solvents could alter the toxicity of commercial preparation.

The aim of the study was to identify the potential hazard of imidacloprid and its commercial formulation Confidor SL 200 to aquatic environment by the assessment of their toxicity using a battery of test organisms, stability and ready biodegradability. We also assessed whether the toxicity of Confidor SL 200 is mainly on the account of solvent mixture or active ingredient present in this commercial formulation. A base set of test species from different taxonomic groups, which are most frequently used for toxicity identification of chemicals and biocides, was selected. These include: bacteria *Vibrio fischeri*, algae *Desmodesmus subspicatus,* crustacean water flea *Daphnia magna* and fish *Danio rerio*. In the case of daphnids, sublethal changes, such as the activities of enzymes: cholinesterase (ChE; involved in nerve signal transmission); catalase (CAT; enables the degradation of hydrogen peroxide formed during oxidative stress) and glutathione S-transferase (GST; involved in the biotransformation of xenobiotics) were also evaluated.

2. Materials and methods

2.1. Chemicals

Imidacloprid and Confidor SL 200 were provided by Bayer CropScience AG, Monheim, Germany. A standard stock solution of imidacloprid was prepared in distilled water with no addition of solvents. A commercially available product Confidor SL 200 contains 200 g L^{-1} of active ingredient and some solvents, such as dimethylsulfoxide (38.4%; v/v) and 1-methyl-2-pyrrolidone (37.5%; v/v). Dibasic and monobasic potassium phosphate, 1-chloro-2,4-dinitrobenzene, L-glutathione (reduced form), 5,5′ dithiobis-2-nitrobenzoic acid, sodium hydrogen carbonate, acetylthiocholine chloride, sodium sulphate and ethylenediaminetetraacetic acid were obtained from Sigma (Germany) and HPLC grade acetonitrile from J.T. Baker. BCA Protein Assay Reagent A and BCA Protein Assay Reagent B were purchased from Pierce (USA). All chemicals were of the highest commercially available grade, typically 99% or higher.

2.2. Stability of imidacloprid in distilled water and stream water

To ensure reliable toxicity data, we checked the stability of imidacloprid in distilled and stream water under the same conditions and concentrations as in the toxicity tests (controlled room temperature 21 ± 1 °C, room light illumination). For the purposes of storage, we also checked if the solution of imidacloprid in distilled water is stable in the dark at fridge temperature 3 ± 2 °C.

Imidacloprid solutions were prepared in distilled water in the following concentrations: 0; 8.75; 17.5; 35; 70; 105 and 140 mg L^{-1}. Each solution was aliquoted in five flasks (100 mL), two of them were kept in the dark at fridge temperature (3 ± 2 °C) and the rest three on light at controlled room temperature (21 ± 1 °C). The solid phase extraction (SPE) of imidacloprid from distilled water solutions was performed immediately after the experiment set up (0 d), and 1, 2, 3, 7, 10, 14, 17 and 22 d from the experiment outset. The SPE extraction with methanol used for the stability studies yielded the extraction recoveries of (95 ± 10) % for imidacloprid. For quantification purposes a calibration curve in the concentration range from 5 ppm to 150 mg L^{-1} was prepared. The *r*-square values for regression line was $r^2 = 0.998$. All determinations were performed in six (for the calibration curve and the experiments in the sunlight) and four (for the experiments in the dark) with relative errors of 5–15%.

Imidacloprid solutions were prepared also in local stream water (pH 8.4, total hardness 140 mg CaO/L, alkalinity 131 mg CaO/L), which was used for fish acute toxicity tests. The stability of 215, 230, 245, 260 and 280 mg L^{-1} of imidacloprid was checked right after the experiment set up (0 d) and at the end of it (after 4 d). Imidacloprid water samples (1 mL) were taken in duplicates.

2.2.1. Sample preparation and HPLC-DAD analysis

Imidacloprid extraction was performed on Strata C18-E columns (100 mg) according to Baskaran et al. (1997). The columns were initially preconditioned with 5 mL of methanol followed by 5 mL of distilled water. Imidacloprid water sample (1 mL) was loaded on the column and the retained imidacloprid was eluted with 2 mL of methanol. In the next step methanol was removed by rotary (Büchi–Rotavapor R-124, Flawil, Switzerland) evaporation in vacuum (T = 30 °C) (Büchi–Waterbath B-480; Germany, Flawil, Switzerland) and dried leftover was rediluted in 1 mL of acetonitrile–water (20:80 v/v) solution (HPLC solvent mixture). Prepared samples were stored at 4 °C until subjected to HP 1000 Series liquid chromatograph (HPLC) equipped with diode array detection (DAD) as described previously (Baskaran et al., 1997). All HPLC-DAD analyses were performed in duplicates on Zorbax C8 (4.6 × 250 mm, 5 μm particle size) column at 25 °C using an isocratic separation with mobile phase of acetonitrile–water (20:80 v/v) at a flow rate 1.25 mL min^{-1}. The stability of imidacloprid was followed from the imidacloprid peak areas at 270 nm, which was identified on the basis of retention time comparison with authentic standard.

2.3. Toxicity tests

At least one preliminary and two definitive trials for each test species were conducted. In each definitive toxicity experiment five concentrations and a control in two replicates were tested. In the case of Confidor SL 200, the solvents listed on the data sheet provided by the supplier (38.4%; dimethylsulphoxide, and 37.5%; v/v 1-methyl-2-pyrrolidone) (further referred to as solvent mixture) at the concentrations used in each toxicity test were tested to investigate the possible toxic effects of the solvents.

2.3.1. Toxicity to bacteria

Luminescence of *V. fischeri* NRRL-B-11,177 was measured using a LUMIStox 300 luminometer (Dr. Lange GmbH, Düsseldorf, Germany). Reactivated liquid-dried bacteria were exposed to 0.78; 1.56; 3.13; 6.25; 12.5; 25; 50; and 100 mg L^{-1} of imidacloprid; 0.016%; 0.031%; 0.063%; 0.13%; 0.25%; and 0.5% (v/v) of Confidor SL 200, and 0.0313%; 0.0625%; 0.125%; 0.25% and 0.5% (v/v) of solvent mixture for 30 min at 15 ± 0.2 °C on a temperature-controlled block (ISO 11348-2, 1998). The percentage of luminescence inhibition was calculated for each concentration relative to the control.

2.3.2. Toxicity to algae

The green, unicellular algae *D. subspicatus* Chodat 1926 (CCAP 276/22; Culture Collection of Algae and Protozoa, Cumbria, United Kingdom) were cultured according to Jaworski (Thompson et al., 1988) on an orbital shaker at 150 rpm (alternately 15 min agitation and resting) at a constant room temperature of 21 ± 1 °C, and fluorescent illumination (4000 lux). In the toxicity tests, the flasks were agitated permanently at 150 rpm and 7000 lux. The algal density and growth rate were determined after 72 h by counting the algal cells in a Bürker counting cell. The tested concentrations

of imidacloprid were 100; 144; 207; 299; and 430 mg L^{-1} and 0.001%; 0.005%; 0.01%; 0.05%; and 0.1% (v/v) of Confidor SL 200, and 0.001%; 0.005%; 0.01%; 0.05% and 0.1% (v/v) of solvent mixture. The inhibition of specific growth rates for each concentration was calculated in comparison to the control (ISO 8692, 2004).

2.3.3. Toxicity to daphnids

Water fleas *D. magna* Straus 1820 were obtained from the Institut für Wasser, Boden und Lufthygiene, des Umweltbundesamtes (Berlin). They were cultured in 2.5 L of modified M4 media (Kühn et al., 1984) at 21 ± 1 °C and 16:8 h light/dark regime (1800 lux) with a diet of the algae *D. subspicatus* Chodat 1926 corresponding to 0.13 mg carbon/daphnia per day.

2.3.3.1. Acute toxicity to daphnids. In the acute toxicity tests, neonates less than 24 h old, derived from the second to fifth brood, were exposed to 10, 40, 70, 100, 130 mg L^{-1} of imidacloprid and 0.0025%; 0.005%; 0.01%; 0.02%; and 0.04% (v/v) of Confidor SL 200, and 0.05%; 0.1%; 0.25%; 0.5% and 1% (v/v) of solvent mixture. After a 24 h and 48 h exposure period the immobile daphnids were counted (ISO 6341, 1996). On the basis of the 48-h EC_{10} and EC_{50} values determined in these range finding tests, the concentrations for further toxicity tests followed by enzyme analyses were selected.

2.3.3.2. Sublethal effects on daphnids after acute exposure. After the acute (48 h) exposure of water fleas sublethal effect of imidacloprid and Confidor SL 200 were studied by measuring their effects on the activities of ChE, GST and CAT. Namely, five test containers containing 20 daphnids/50 mL of test solution were prepared for each concentration of imidacloprid (10, 20, 30 and 40 mg L^{-1}). After a 48-h exposure period, the immobile daphnids were counted, removed, and all mobile animals (70–100) were combined into one sample. Each acute toxicity test was repeated three times.

The animals were homogenized for 3 min in 0.7 mL of homogenization buffer (50 mM phosphate buffer pH 7.0), using a glass–glass Elvehjem–Potter homogenizer. The excess imidacloprid was removed from the homogenizer and the surface of the animals by rinsing three times with 2 mL of the homogenization buffer combined with 5 mM EDTA. The homogenate was centrifuged for 15 min at 15000*g* and 4 °C (Jemec et al., 2007).

ChE activity was determined according to Ellman et al. (1961), and Jemec et al. (2007) using microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS). The reaction mixture was prepared in 100 mM potassium phosphate buffer pH 7.3 containing acetylthiocholine chloride and 5,5′ dithiobis-2-nitrobenzoic acid in the final concentrations of 1 mM and 0.5 mM, respectively. Protein supernatant (100 μL) was added to start the reaction, which was followed spectrophotometrically at 412 nm and 25 °C for 15 min.

GST activity was determined using the method described by Habig et al. (1974) and Jemec et al. (2007), using microtiter plates (Bio-Tek® Instruments, USA; PowerWave™ XS) and 1-chloro-2,4-dinitrobenzene as a substrate. The final reaction mixture contained 1 mM of 1-chloro-2,4-dinitrobenzene and 1 mM of reduced glutathione. 50 μL of protein supernatant were added to start the reaction. The reaction was followed spectrophotometrically at 340 nm and 25 °C for 3 min.

CAT activity was determined according to Aebi (1984). We added 50 μL of protein supernatant to 750 μL of H_2O_2 solution (10.8 mM) prepared in 50 mM potassium phosphate buffer pH 7.0. The reaction was followed spectrophotometrically at 240 nm and 25 °C for 5 min on a Shimadzu UV-2101PC spectrophotometer (Japan). The concentrations of substrates used for all enzymes were saturating and ensured the linear changes of absorbance with time and the concentration of proteins.

One enzyme unit (EU) was determined as the amount of ChE that hydrolyses 1 nmole of acetylthiocholine/min (ε_{412} = 13,600 M^{-1} cm^{-1}), the amount of CAT that degrades 100 μmoles of hydrogen peroxide/min (ε_{240} = 43.6 M^{-1} cm^{-1}), and the amount of GST that conjugates 100 nmoles of reduced glutathione/min (ε_{340} = 9600 M^{-1} cm^{-1}). These enzyme units were chosen to facilitate comparison of all enzyme activities for each chemical.

Protein concentration was determined using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

2.3.4. Toxicity to fish

2.3.4.1. Zebrafish survival. Specimens of zebrafish *Danio rerio* Hamilton Buchanan, obtained from a commercial supplier, were initially acclimated to the test conditions in water obtained from an unpolluted stream (pH 8.4, total hardness 140 mg CaO/L, alkalinity 131 mg CaO/L) 7 d prior to the experiment. They were fed daily with commercial fish food and illuminated with fluorescent bulbs for 12 h per day.

During the toxicity tests, the animals were placed in 2.5 L of slightly aerated test solution at 21 ± 1 °C (ISO 7346-1, 1996). Dead fish were counted and removed from the tanks daily during a 96 h exposure period. The concentration of oxygen in the test solutions was measured at the beginning and end of the experiment using an oxygen electrode (WTW Oximeter, OXI 96). The percentage of mortality for each tested concentration of Confidor SL 200 (0.075%; 0.1%; 0.11%; and 0.13%; v/v) and 200; 215; 260; 280; and 300 mg L^{-1} of imidacloprid, and 0.075%; 0.1%; 0.11%; and 0.13% (v/v) of solvent mixture was calculated after 24, 48, 72, and 96 h of exposure.

2.3.4.2. Zebrafish embryo test. A detailed description of zebrafish breeding to obtain eggs was published by Kammann et al. (2004). Briefly, adult zebrafish were bred in a temperature-controlled room in aquarium (60 × 30 × 30 cm) containing 45 L of tap water with constant temperature (26 °C) and photoperiod (12 h light:12 h dark). Filtration was provided by internal bioactive filter device. Fish were fed three times daily with commercially available dried fish food (Nutrafin, Tetramin). A day before breeding a plastic spawning box covered with stainless steel mesh was placed in the breeding tank. On the following day, one hour after the light cycle started, the spawning plastic box was removed from the tank and eggs were collected and rinsed with synthetic medium prepared according to ISO 15088 (2007).

The toxicity test was performed according to the same ISO standard. Fertilized eggs in the four to eight cell stages were placed in 24-well plates; each well contained 1 mL of synthetic ISO medium with different concentrations of imidacloprid (10, 40, 60, 80, 160 and 320 mg L^{-1}); Confidor SL 200 (0.1%, 0.2%, 0.4%, 0.6% and 0.8%; v/v), and 0.3%; 0.4%; 0.5% and 0.6% (v/v) of solvent mixture. For each experiment a control containing only synthetic ISO medium was prepared. After 24 h and 48 h of exposure at 26 °C lethal malformations, i.e. egg coagulation, missing heartbeat, missing somites, missing tail detachment from the yolk sac, and non-lethal malformations, i.e. no eye and body pigmentation, missing blood flow, spine deformation, yolk sac edema, incomplete eye and ear development were observed. The percentages of each malformation were calculated for the exposed concentrations of imidacloprid and Confidor SL 200. The reference chemical 3,4-dichloroaniline (2, 2.5 and 3.7 mg L^{-1}) was used as a positive control. After 48 h of exposure 2 mg L^{-1} of 3,4-dichloroaniline caused the changes of the majority of endpoints in 10% of specimens, at 2.5 mg L^{-1} in 30% of specimens, while at 3.7 mg L^{-1} of the reference chemical, from 30% to 100% of the specimens were affected when different end-points were evaluated. Based on this, the tests fulfilled the validity criteria prescribed by the standard (ISO 15088,

2007), which states, that at least one effect at 3.7 mg L^{-1} of 3,4-dichloroaniline should be observed in more than 10% of specimens. We consider the later validity criteria very broad, and recommend that either concentration 2 mg L^{-1} or 2.5 mg L^{-1} be rather used as a reference concentration.

2.4. Biodegradability

Prior to imidacloprid biodegradation test, its toxicity to a mixed bacterial community was assessed. The activated sludge microorganisms (the final concentration was 150 mg L^{-1} of suspended solids) from the aeration tank of the municipal laboratory waste water treatment plant were exposed to increasing concentrations of imidacloprid according to ISO 8192 (1986). Oxygen consumption was measured with an oxygen electrode (WTW Oximeter, OXI 96) following biochemical degradation of meat extract, peptone, and urea every 30 min during 3 h. The inhibition of oxygen consumption rate compared to the control was determined for imidacloprid (100, 150, 200, 300 and 400 mg L^{-1}). Based on these preliminary results, the biodegrability of Confidor SL 200 was not tested due to extensive consumption of oxygen as a result of solvents degradation.

The aerobic biodegradability of imidacloprid was studied in a closed respirometer (Baromat, WTW, BSB-Messgerät, Model 1200). The same source of activated sludge was used as in a toxicity test with mixed bacterial community; concentration 30 mg L^{-1} of suspended solids was used. The oxygen consumption was measured during 28 d or until the plateau was reached (ISO 9408, 1991) in the samples containing 250 and 450 mg L^{-1} of imidacloprid.

2.5. Statistical analyses

2.5.1. Bacteria

The 30 min IC_{20}, IC_{50} with 95% confidence limits and IC_{80} values for luminescence bacteria were calculated using a linear regression analysis supported by computer software (Dr. Bruno Lange, 2000). The IC_{20} was considered a toxicity threshold. In a case of mixed bacterial community the percentages of inhibition of oxygen consumption were plotted against corresponding concentrations of imidacloprid on semi-logarithmic paper and the IC_{20}, IC_{50}, and IC_{80} values were determined using linear regression analysis. The IC_{20}, IC_{50}, IC_{80} stand for inhibition concentration that causes 20%, 50% and 80% inhibition of luminescence or oxygen consumption compared to the control.

2.5.2. Algae

The percentages of inhibition of specific growth rates were plotted against concentration on semi-logarithmic paper and the 72 h IC_{10}, IC_{50}, and IC_{90} values (inhibition concentrations that cause 10%, 50% and 90% inhibition of algal growth in comparison to the control, respectively) were estimated using linear regression analysis.

2.5.3. Daphnids and fish

The percentages of immobile daphnids, fish lethal and sublethal end-points were analysed with probit analysis to determine the effective (EC_{10}, EC_{50}, EC_{90}) and lethal (LC_{10}, LC_{50}, LC_{90}) concentrations that cause 10%, 50% and 90% of daphnids immobility, fish dead or sublethal effects, respectively. The 95% confidence limits are provided for the EC_{50} (LC_{50}) values (US EPA, 1994).

2.5.4. Enzyme analyses

The effects of the imidacloprid on enzymes were compared by Kruskal–Wallis analysis and non-parametric Mann–Whitney *U* test ($P < 0.05$), using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics Corporation). Homogeneity of variance was tested using Levene's test. The percentages given in the results represent the change in medians of ChE, GST and CAT activity in exposed animals compared to control.

2.5.5. Biodegradability

Biodegradation curves were plotted as the percentages of biodegradation for each sample of imidacloprid versus time. A final level of biodegradation, a lag phase and a degradation time were the parameters used for biodegradability assessment.

3. Results and discussion

3.1. Stability of imidacloprid in distilled and stream water

The results of HPLC-DAD measurements have shown the same levels of imidacloprid at any of the tested concentrations when stored in the dark at fridge temperature for 22 d (Fig. 1a).

The stability of imidacloprid solution stored at room light and 21 ± 1 °C depended on the concentration of imidacloprid. For example, the concentrations of imidacloprid up to 70 mg L^{-1} did not change during 22 d, while the highest tested concentrations 105 mg L^{-1} and 140 mg L^{-1} of imidacloprid in the same period decreased by 16% and 24% in comparison to their initial concentrations (Fig. 1b). This could be explained by the presence of sunlight. Slight variations of imidacloprid levels were noticed at higher concentrations (70, 105 and 140 mg L^{-1}) until day three. This variability is probably the result of an experimental error.

The concentrations of imidacloprid measured in the stream water from the fish toxicity tests at the beginning of the experiment were slightly lower (up to 5%) as initial values. Instead of 215, 230, 245, 260 and 280 mg L^{-1} of imidacloprid, the following levels were measured: 216, 216, 232, 250 and 270, respectively.

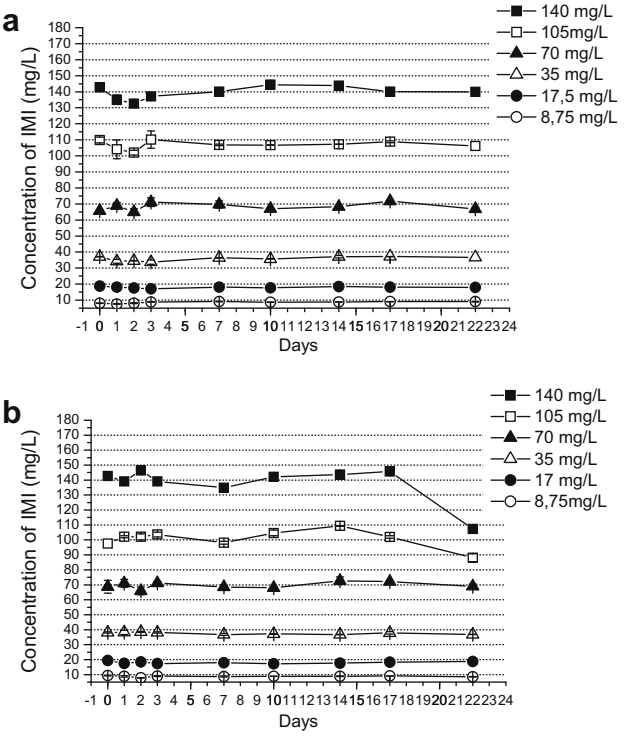

Fig. 1. The effect of storage conditions: (a) dark and fridge temperature (2–5 °C); (b) light and room temperature (21 ± 1 °C) on the stability of imidacloprid in distilled water (mean of six (Fig. 1a) and four replicates (Fig. 1b) ± standard error of mean).

The concentrations of imidacloprid were stable during the experiment (up to 4 d).

Different literature data are available on the stability of imidacloprid in aqueous medium. Similarly as in our study, Overmyer et al. (2005) reported that imidacloprid was stable during 48 h of toxicity tests using aquatic insects *Simulium vittatum* (20 °C, 16:8-h light:dark period). Several studies reported the stability of imidacloprid under simulated environmentally relevant conditions. Namely, Kagabu and Medej (1995) determined a short half live of imidacloprid (1–3 h) when exposed to simulated sunlight (250 W at 30 °C). On the contrary, Sarkar et al. (1999) reported longer half lives (31–43 d) of commercial preparation Confidor SL 200 depending on the temperature and pH.

3.2. Toxicity tests

The toxicity values for analytical grade imidacloprid, Confidor SL 200, the amount of imidacloprid in Confidor SL 200 and solvent mixture in this formulation are provided in Fig. 2 and Tables 1–3. We compare the toxicity of analytical grade imidacloprid to its commercial formulation Confidor SL 200 for each species and assess whether the toxicity of Confidor SL 200 is mainly on the account of solvent mixture or imidacloprid present in Confidor SL 200.

3.2.1. Acute toxicity to bacteria, daphnids and zebrafish

Analytical grade imidacloprid was similarly toxic to *V. fischeri* as imidacloprid formulated as Confidor SL 200. Also, the solvent mixture alone was significantly less toxic than Confidor SL 200. This indicates, that the toxicity of Confidor SL 200 to *V. fischeri* is mainly due to imidacloprid action, and not because of solvents (Fig. 2a, Table 1) There are no other reported data concerning the toxicity of imidacloprid to aquatic bacteria (SERA, 2005).

When imidacloprid was formulated as Confidor SL 200, it was more toxic to daphnids than analytical grade imidacloprid. Also, Confidor SL 200 was significantly more toxic than the solvents alone (48 h EC_{50} of Confidor SL 200 was 20 times lower). Namely, when the amount of solvent mixture, contained in the highest tested concentrations of Confidor SL 200 was tested, no toxicity to daphnids was observed. This implies, that the toxicity to daphnids cannot be attributed either to solvents or imidacloprid alone, but a combination of both increases the toxicity of this commercial formulation in comparison to analytical grade imidacloprid (Fig. 2b, Table 1).

The 48 h EC50 obtained for *D. magna* in our research was 56.6 mg L^{-1} of imidacloprid, which is in the range of the literature data reported: 48 h LC50 and the 48 h EC50 values obtained for *D. magna* were 17.36 mg L^{-1} (Song et al., 1997) and 85 mg L^{-1} (Young and Blakemore, 1990; SERA, 2005), respectively. Imidacloprid impairs the nerves function and consequently the normal mobility

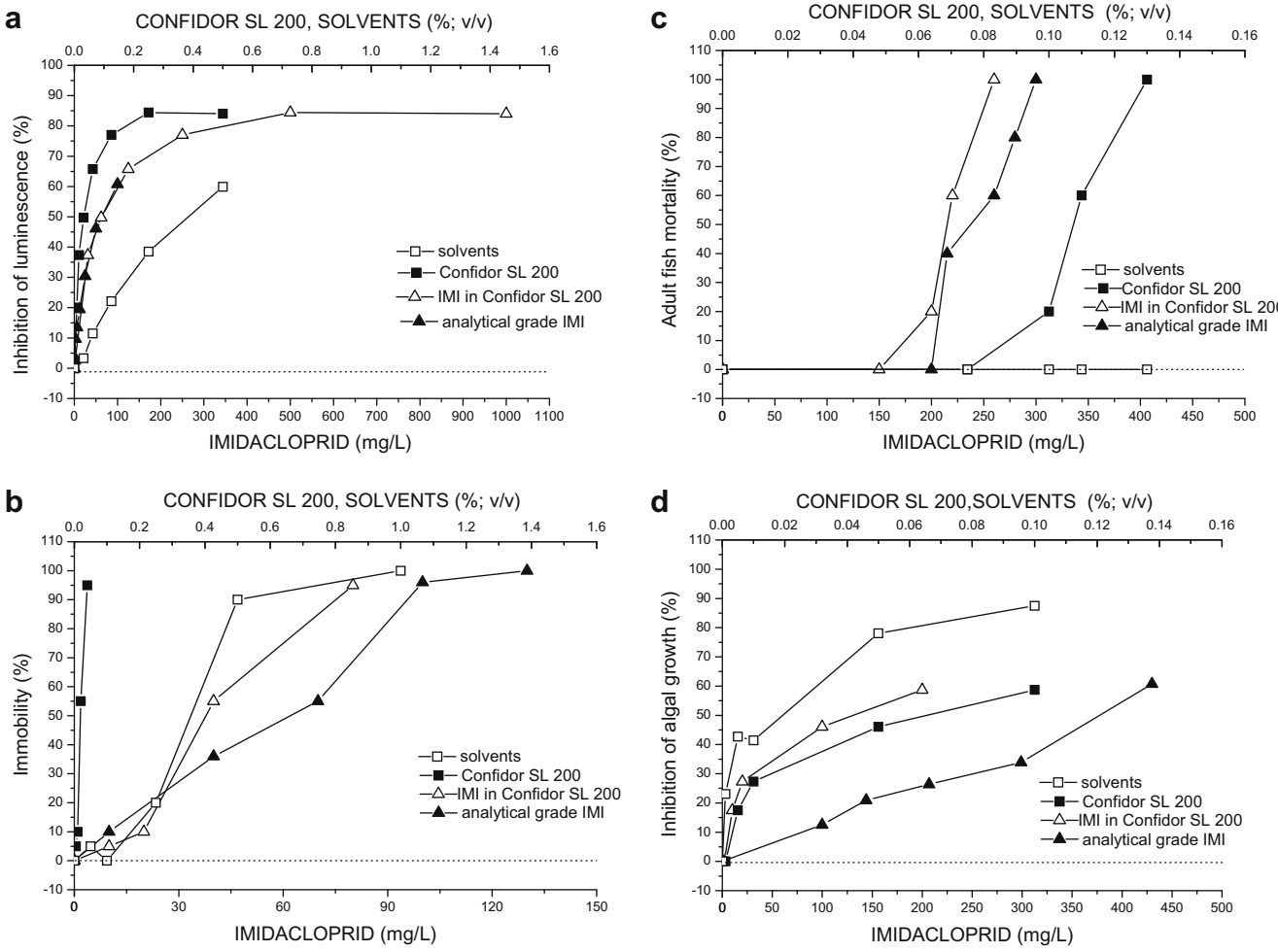

Fig. 2. Toxicity of imidacloprid and Confidor SL 200 to (a) *Vibrio fischeri*, (b) *Daphnia magna*, (c) adult *Danio rerio* and (d) algae *Desmodesmus subspicatus*. The lower *x*-axis (in mg L^{-1}) stands for analytical grade imidacloprid and the concentration of imidacloprid in Confidor SL 200. The upper *x*-axis (in %) stands for solvents and Confidor SL 200. The concentrations of imidacloprid in Confidor SL 200 applied on lower *x*-axis do not correspond to concentrations of Confidor SL 200 on upper *x*-axis.

Table 1
ECx/ICx/LCx values (effective, inhibition and lethal concentrations) of imidacloprid and Confidor SL 200 to *Daphnia magna*, *Vibrio fischeri* and adult *Danio rerio*.

Species	IMI[a] (mg L^{-1})		Confidor SL 200 (%; v/v)		IMI[b] (mg L^{-1})	
D. magna	24 h	48 h	24 h	48 h	24 h	48 h
EC_{10}	36.8	22.5	0.011	0.008	22	12
EC_{50}	97.9	56.6	0.019	0.018	38	30
(95% CL)	(81.4–127.7)	(34.4–77.2)	(0.016–0.024)	(0.014–0.022)	(32–48)	(28–44)
EC_{90}	260	142	0.035	0.038	70	70
V. fischeri	30 min		30 min		30 min	
IC_{20}	11.9		0.0056		11.2	
IC_{50}	61.9		0.028		56	
(95% CL)	(61.9–62.0)		(0.015–0.041)		(30–82)	
IC_{80}	320		0.140		280	
D. rerio	96 h		96 h		96 h	
LC_{10}	201		0.097		194	
LC_{50}	241		0.107		214	
(95% CL)	(224–257)		(0.101–0.115)		(202–230)	
LC_{90}	290		0.118		236	

[a] Analytical grade imidacloprid.
[b] Concentration of IMI in corresponding %, v/v Confidor SL 200 solution, CL – corresponding 95% confidence limits.

Table 2
LCx/ECx (lethal and effective concentrations) of imidacloprid, Confidor SL 200 and solvent mixture used in Confidor SL 200 based on the development of zebrafish embryos after 48 h.

	Danio rerio – development of embryos (48 h) Confidor SL 200 (%; v/v)								
	Egg coagulation[b]			Missing heartbeat[b]			Missing tail detachment[b]		
	Confidor SL 200 (%; v/v)	IMI[a] (mg L^{-1})	Solvents[d] (%; v/v)	Confidor SL 200 (%; v/v)	IMI[a] (mg L^{-1})	Solvents (%; v/v)	Confidor SL 200 (%; v/v)	IMI[a] (mg L^{-1})	Solvents (%; v/v)
LC_{10}	0.442	884	0.228	0.150	300	0.237	0.406	812	0.254
LC_{50}	0.580	1160	0.452	0.251	502	0.350	0.575	1150	0.400
(95% CL)	(0.500–0.658)	(1000–1316)	(0.314–0.758)	(0.194–0.315)	(388–630)	(0.261–0.404)	(0.486–0.668)	(972–1336)	(0.311–0.472)
LC_{90}	0.762	1524	0.896	0.418	836	0.517	0.814	1628	0.631
	Missing somites[b]			Missing eye pigmentation[c]			Missing body pigmentation[c]		
LC/EC_{10}	0.172	344	0.287	0.174	348	0.196	0.160	320	0.166
LC/EC_{50}	0.413	826	0.445	0.366	732	0.419	0.313	626	0.368
(95% CL)	(0.307–0.553)	(614–1106)	(0.222–0.560)	(0.275–0.466)	(550–932)	(0.142–0.672)	(0.236–0.394)	(472–788)	(0–0.487)
LC/EC_{90}	0.993	1986	0.689	0.767	1534	0.894	0.613	1226	0.812
	Missing blood flow[c]			Incomplete eye development[c]			Incomplete ear development[c]		
EC_{10}	0.111	222	0.237	0.181	362	0.248	0.168	336	0.150
EC_{50}	0.204	408	0.350	0.380	760	0.423	0.313	626	0.284
(95% CL)	(0.154–0.262)	(308–524)	(0.261–0.404)	(0.287–0.485)	(574–970)	(0.320–0.523)	(0.238–0.391)	(476–782)	(0.009–0.363)
EC_{90}	0.373	746	0.517	0.799	1598	0.717	0.585	1170	0.537

[a] Concentration of IMI in corresponding % (v/v) of Confidor SL 200 solution.
[b] Lethal endpoints.
[c] Sublethal endpoints, CL – corresponding 95% confidence limits.
[d] Solvents refer to solvent mixture used in Confidor SL 200 solution.

Table 3
Chronic toxicity of imidacloprid and Confidor SL 200 to *Desmodesmus subspicatus* and *Daphnia magna* (Jemec et al., 2007).

Test species	IMI[b] (mg L^{-1})	Confidor SL 200 (%; v/v)	IMI[c] (mg L^{-1})
D. subspicatus			
72 h IC_{10}	106	2.8×10^{-3}	5.6
72 h IC_{50}	389	5.8×10^{-2}	116
72 h IC_{90}	1425	1.18	2351
Daphnia magna[a]			
21 d LOEC	2.50	2.5×10^{-3}	5.0
21 d NOEC	1.25	1.25×10^{-4}	2.5

[a] Jemec et al. (2007).
[b] Analytical grade imidacloprid.
[c] Concentration of IMI in corresponding % (v/v) of Confidor SL 200 solution.

of organisms, which is the most frequent observed endpoint of the acute toxicity test with water fleas. In comparison to some other pesticides, e.g. diazinon, imidacloprid is not highly toxic to daphnids (Jemec et al., 2007). On the contrary, some invertebrate species revealed high sensitivity to imidacloprid; the highest toxicity was observed for *Hyalella azteca* and *Chironomus tentans* with the corresponding 96 h LC50 values 0.526 mg L^{-1} and 0.0105 mg L^{-1}, respectively (SERA, 2005).

When imidacloprid was formulated as Confidor SL 200, it was slightly more toxic to adult zebrafish than analytical grade imidacloprid. When the amount of solvent mixture, contained in the highest tested concentrations of Confidor SL 200 (0.13%; v/v) was tested, no toxicity to adult fish was observed. Again, as in the case of daphnids, the combination of active ingredient imidacloprid and solvents increase the toxicity of commercial formulation (Fig. 2c, Table 1)

No toxicity of analytical grade imidacloprid to development of zebrafish embryos was observed even at 320 mg L^{-1}. However Confidor SL 200 revealed high toxicity to all observed endpoints; the most sensitive was found to be blood circulation and heartbeat comparing the obtained LC50/EC50 values. The toxic effects of

solvent mixture used in Confidor SL 200 on embryos were similar to Confidor SL 200 (Table 2). This indicates that probably the toxicity of this commercial preparation to zebrafish embryos is mainly on the account of solvents.

The survival of adult zebrafish exposed to Confidor SL 200 was more affected than the embryos development comparing the LC50/EC50 values (Tables 1 and 2). Literature review indicated that the sensitivity of adult and embryos of zebrafish depends on tested chemical and its mode of toxic action (Lange et al., 1995; Roex et al., 2002; Kammann et al., 2006). No previous data on the toxicity of imidacloprid to zebrafish are available. Our result is similar to those reported to golden ide *Leuciscus idus melanotus* as the 96 h LC50 was obtained at 237 mg L^{-1} (Pfeuffer and Matson, 2001). The reported 96 h LC50s for rainbow trout *Oncorhynchus mykiss* and common carp *Cyprinus carpio* were 211 mg L^{-1} and 280 mg L^{-1}, respectively (SERA, 2005; Fossen, 2006).

The comparison of acute toxicity values (Tables 1 and 2) for different species showed, that imidacloprid and Confidor SL 200 were found to be the most acutely toxic to daphnids, followed by bacteria *V. fischeri* and zebrafish adults and embryos.

3.2.1.1. Effects on enzyme activities. The activities of ChE, GST and CAT did not change during acute exposure of daphnids to imidacloprid or Confidor SL 200. The values of ChE, CAT and GST activities in control animals were 3.48 ± 0.13; 1.29 ± 0.049 and 1.42 ± 0.036 EU/mg protein, respectively in the case of imidacloprid and 3.03 ± 0.38, 1.15 ± 0.09 and 1.33 ± 0.047 EU/mg protein in the case of Confidor SL 200. This suggests that these enzyme activities are not an early, sensitive biomarker of exposure to imidacloprid or Confidor SL 200. Similarly was shown in our previous work (Jemec et al., 2007), where the activities of the same enzymes were decreased in daphnids chronically exposed up to 40 mg L^{-1} of imidacloprid and 0.02% Confidor SL 200, but these changes were shown to be due to generally impaired physiological state of an organism and not specific action of imidacloprid and Confidor SL 200. Only one study was previously published on the acute effects of imidacloprid on ChE and GST activities, where no changes of the latter were found in earthworms exposed up to 1 mg L^{-1} of imidacloprid (Capowiez et al., 2003).

3.2.2. Chronic toxicity to algae and daphnids

The results of chronic toxicity tests with algae and daphnids are given in Fig. 2d and Table 3. The 72 h IC50 value obtained for *D. subspicatus* was 389 mg L^{-1} indicating the lowest toxicity of imidacloprid observed among the selected tested organisms. It was found that the imidacloprid in Confidor SL 200 was much more toxic than active ingredient alone. Solvents contributed a major part to toxicity for algae, because the tested solvents alone inhibited the algal growth already at 0.005 v/v%. Literature data showed that the highest tested concentrations in toxicity tests (10 mg L^{-1} and 119 mg L^{-1} of analytical grade imidacloprid) did not cause adverse effects on *D. subspicatus* and *Selenastrum capricornutum* (SERA, 2005). Diatom *Navicula pelliculosa* was found to be the most sensitive algal species as the 4 d NOAEC and the LOAEC were 6.69 mg L^{-1} and 9.88 mg L^{-1} of imidacloprid, respectively (SERA, 2005).

In our laboratory, the highest toxicity of imidacloprid among the species tested in the present study was previously reported on the reproduction of daphnids: the 21 d NOEC was 1.25 mg L^{-1} of imidacloprid (Jemec et al., 2007). Contrary to the acute toxicity observations with daphnids, bacteria, and zebrafish, the toxicity of imidacloprid to the reproduction of daphnids did not increase when testing the Confidor SL 200. The obtained 21 d NOEC was even higher as those obtained for pure chemical. Similar result was reported by Young and Blakemore (1990) as they determined the 21 d NOAEC at 1.8 mg L^{-1} of imidacloprid using the immobility as endpoint. Also data for other aquatic crustaceans show high toxicity of imidacloprid, i.e. the NOAEC for *Mysidopsis bahia* was found at 0.000163 mg L^{-1} after the chronic exposure (SERA, 2005).

At the moment, imidacloprid is not regularly monitored in aquatic environments. Very few data are available and they indicate low environmental levels of imidacloprid; the lowest and the highest measured environmental concentrations were 1 μg L^{-1} and 14 μg L^{-1} of imidacloprid (Pfeuffer and Matson, 2001; US Geological Survey, 2003). These concentrations are lower than chronic levels observed for daphnids. However, some local point-source contamination which can occur as a consequence of an accidental spill could pose a potential chronic risk to *D. magna* according to the results obtained in our study. Moreover, the acute risk for more sensitive crustacean species than daphnids, e.g. *Hyalella azteca* and *Chironomus tentans* exists (SERA, 2005).

3.3. Ready biodegradability

Initially, acute toxicity of imidacloprid was determined using activated sludge to eliminate possible inhibition of biodegradation due to potential toxicity of imidacloprid to microorganisms. Imidacloprid was non-toxic to mixed bacterial community of activated sludge as the inhibition of oxygen consumption at the highest concentration tested (400 mg L^{-1}) was 6% compared to the control. In the case of Confidor SL 200 toxicity to activated sludge could not be evaluated due to intensive degradation of the solvents present in the Confidor SL 200.

The samples with 250 and 450 mg L^{-1} of imidacloprid were tested for biodegradability. Tested samples were non-toxic to microorganisms and biodegradation started immediately without a lag phase. The final levels of biodegradation were between 9% and 12%. The samples containing 250 and 450 mg L^{-1} of imidacloprid were not readily biodegradable according to the recommendations for the ready biodegradability classification of pure chemicals as the "pass level" of biodegradation in the O_2 test was not achieved (Struijs and van den Berg, 1995). The obtained persistence is in agreement with the statement that imidacloprid is likely to remain in water column in aquatic systems (Overmyer et al., 2005). The degradation and elimination of imidacloprid was investigated in water–sediment systems (Spiteller, 1993; Krohn and Hellpointner, 2002). It was found that radioactively labelled imidacloprid disappeared quickly from the water phase to the sediment phase. At the same time formation of CO_2 by microbes due to mineralization was observed throughout the experiments although its proportion was quite low (0.7–2.0%) and the process was slow. The calculated DT50 values (time after which half of the initial concentration of imidacloprid was disappeared) were 30 d for elimination from the water phase and between 130 and 160 d for different types of sediments. Henneböle (1998) demonstrated that the DT50 was reduced to some days under the influence of sunlight using a water–sediment system. It was also reported that the elimination of imidacloprid was lower in the oligotrophic system (Bayer, 2000) contrary to the fast disappearence in eutrophic conditions. In our experiment, oligotrophic system with low concentration of microorganisms and nutrients was used and consequently the low biodegradability of imidacloprid was determined.

4. Conclusions

The results of this study show that imidacloprid is not highly toxic to tested aquatic organisms in comparison to some other environmental pollutants tested in the same experimental set-up (Tišler and Zagorc-Končan, 2002; Tišler et al., 2004; Tišler and Kožuh Eržen, 2006). Water fleas *D. magna* were the most sensitive

species after a short-term (48 h EC50 = 56.6 mg L^{-1}) and long-term exposure (21 d NOEC = 1.25 mg L^{-1}) followed by *V. fischeri*, zebrafish and algae. The activities of enzymes ChE, GST and CAT of daphnids were not early, sensitive biomarkers of exposure to imidacloprid and its commercial product. Imidacloprid was found persistent in water samples and not readily biodegradable in aquatic environment. The toxicity of commercial formulation Confidor SL 200 was intensified in comparison to the analytical grade imidacloprid to daphnids, zebrafish and especially in a case of algae the solvents highly elevated the adverse effects. Therefore, due to the increased predicted use of commercial products containing imidacloprid in the future and the obtained findings of this study we recommend additional toxicity and biodegradability studies of other commercial products containing imidacloprid as an active ingredient in the aquatic environment. Only, these studies will provide the final answer, whether imidacloprid is an appropriate substitution for other more toxic pesticides.

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