Aquatic Toxicology 74 (2005) 285–293

AQUATIC TOXICOLOGY

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Unexpected effects of zinc pyrithione and imidacloprid on Japanese medaka fish (*Oryzias latipes*)

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Received 25 January 2005; received in revised form 25 May 2005; accepted 10 June 2005

Abstract

Biological effects of the biocide zinc pyrithione (Zpt), used in anti-dandruff shampoos and antifouling paints and the agricultural insecticide imidacloprid on Japanese medaka fish (*Oryzias latipes*) were assessed in experimental rice fields. Both chemicals are toxic to medaka, in particular Zpt, which also causes teratogenic effects such as spinal cord deformities in embryos at very low, sublethal concentrations. Rates of malformation in medaka fry from paddies treated twice a week with anti-dandruff shampoo (0.18–0.37 μL/L each time) over a period of 4 months were within the natural background, perhaps due to the quick dissipation rate of this chemical in the environment. Both Zpt and imidacloprid caused stress syndrome in juvenile medaka, with fish from Zpt-shampoo fields having a significantly lower weight to body length ratio than those from control fields. As it often happens with stressed fish, a massive infestation by a *Trichodina* ectoparasite was observed in medaka from imidacloprid fields. However, despite their high stress levels, fish from the Zpt fields did not suffer such infestation, supposedly because the disinfectant action of this biocide.

Keywords: Rice; Biocides; Chronic exposure; Physiological stress; Fish parasites

1. Introduction

Rice cultivation requires the flooding of fields between planting of seedlings and fructification, a period usually lasting 3–4 months. During that time, several fish species may inhabit the paddy and adjacent drains (Heckman, 1979), and be subjected to the effects of pesticides applied routinely to the crop. Apart from the acute toxicity of some insecticides that may result in direct mortality, fish are very sensitive to sublethal concentrations of such toxicants and their accompanying surfactants. Fish may be stressed (Sancho et al., 1997), their hatching and development may be delayed (Gormley and Teather, 2003), they may show gill damage (Hofer et al., 1995), swimming impairment, changes in temperature or salinity tolerances (Heath et al., 1997) and malformations (Luckenbach et al., 2001) of several kinds.

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doi:10.1016/j.aquatox.2005.06.003

Table 1
Some physico-chemical properties of zinc pyrithione (Turley et al., 2000) and imidacloprid (Tomlin, 2001–2002), and their half-lives in several media

Property	Zinc pyrithione	Imidacloprid
Molecular weight	317.7	255.7
Water solubility (mg/L, 20 °C)	Insoluble	610
Vapour pressure (mPa, 20 °C)	Not available	4×10^{-7}
Octanol–water partitioning coefficient ($\log K_{ow}$)	9.33	0.57
Half-life		
Aqueous photolysis	<2 min, 4 h[a]	1.2 h[b], 126 min[c]
Freshwater (sunlight)	25 min	4 days[d]
(Dark)	8 h	10–24 weeks[e]
Water and sediment	0.5 h (anaerobic)	66 days[d]
	2–22 h (aerobic)	50–70 days[f]

[a] Thomas (1999).
[b] Moza et al. (1998).
[c] Wamhoff and Schneider (1999).
[d] This study.
[e] Kagabu and Medej (1995).
[f] Hoshino and Takase (1993).

One the most effective insecticides against sucking insect pests currently used in rice crops is imidacloprid (1-[6-chloro-3-pyridylmethyl]-*N*-nitroimidazolidin-2-ylideaneamine), a neurotoxic chemical with the same mode of action as nicotine (Matsuda et al., 2001), which competes with acetylcholine for receptor sites. Unlike most modern insecticides imidacloprid is systemic, i.e. it is absorbed by the rice seedlings and stored in their tissues, and for this reason it is applied in nursery boxes before planting. When an insect comes to feed on the plant, it ingests the insecticide and dies. The insecticidal activity of imidacloprid lasts for about 3 months (Iwaya and Kagabu, 1998), thus protecting the plant from early pests. However, by its very systemic nature imidacloprid moves easily between plant tissues, and also from the roots to the soil beneath and water in the field (Felsot et al., 1998). In this way, imidacloprid is slowly released into the aquatic environment (Nemeth-Konda et al., 2002), where it is very stable to hydrolysis (Kagabu and Medej, 1995) but is decomposed under sunlight (Moza et al., 1998) (Table 1). Although some authors have investigated the toxicity of this chemical to aquatic crustaceans (Song et al., 1997), no studies dealing with the effect of this insecticide on paddy fish are available yet to assess its ecological impact.

Besides pesticides, in many rural areas of Asian countries the effluent from house drains, containing a diverse mixture of detergents, shampoos and other chemicals, is discharged directly into the irrigation channels of rice fields. Although most people do not know it, some ingredients in this cocktail of polluted waters are biocides with an acute toxicity equivalent to that of agricultural insecticides. Such is the case of zinc pyrithione (2-mercapto pyridine-*N*-oxide zinc salt), which has been used in anti-dandruff shampoos in many countries for years, and more recently incorporated in antifouling paints for large boats and ships (Turley et al., 2000) due to the ban on tributyltin (TBT). The deleterious effect of this biocide on embryos of medaka, a common fish in paddies in Japan, has been studied previously (Goka, 1999). In that study, embryos exposed to sublethal concentrations of Zpt (0.003–0.007 mg/L) and two anti-dandruff shampoos that contained the Zpt active ingredient developed spinal cord malformations.

Because it is often difficult to extrapolate from toxicity results obtained in the laboratory to the biological effects occurring in outdoor environments, we decided to study the acute and sublethal effects of Zpt and imidacloprid on juvenile medaka fish (*Oryzias latipes*, orange-red strain) under rice cultivation conditions.

Although both chemicals are toxic to medaka, in particular Zpt, it is also known that photolysis is a significant factor in their degradation in natural environments (Armbrust, 2000; Maraldo and Dahllo, 2004). This prompts the question whether or not the toxicity of imidacloprid and Zpt would be inhibited in natural environments (see Table 1), and if so what effects would be observed in aquatic organisms.

2. Material and methods

Six small experimental fields (5.2 m × 1.6 m) were flooded with bore water to a depth of approximately 4 cm and planted with rice seedlings on May 14, 2004, with an array of 27 × 3 swards per field and leaving a 15-cm embankment on each side. Fields remained flooded for 4 months until the end of the experiment, allowing sufficient observations throughout the entire cultivation and fructification stages of rice. Water from the fields drained to individual culverts, where it was recycled using an automated pumping system that kept water levels constant in the paddies, and ensured that each field remained independent.

Two fields contained rice seedlings, which the day before transplanting had been treated with Admire GR (1% imidacloprid) at a rate of 215 g a.i./ha, which is 1.5 times the recommended rate of application on commercial rice fields. Two more rice fields were treated with a shampoo that contained Zpt (approximately 1–2% a.i.), adding 5 mL per field twice a week to mimic a standard rate of utilization by households in the vicinity. The average volume of water in each field was 270 L, so nominal concentrations of Zpt added each time were 0.185–0.370 μL/L (ppm), while the total amount of shampoo added in each field until the end of the 4-month experiment was 180 mL. The remaining two rice fields were not treated with any chemicals but kept as controls.

Water temperature was similar among fields: it remained at 23 ± 1 °C during the first month; but once the typhoon season was over (end of June), it increased to 28 ± 1 °C and was maintained at this level through most of the summer months, declining in late August to 25 ± 1 °C. Prior to chemical treatment, water pH in all fields was similar (7.08 ± 0.49). The imidacloprid-treated fields had pH values of 8.51 ± 0.76 from June 3rd onwards, but water pH from both the Zpt-treated and control fields were only slightly higher than initial levels (7.56 and 7.43, respectively). Such difference in pH between imidacloprid and the other fields was statistically significant ($P < 0.01$) and related to a green algae bloom (*Spirogyra* sp.) that developed in both imidacloprid fields at the end of May. Interestingly, water in the imidacloprid fields had a very transparent appearance between June and mid-July (20 FTU), contrasting with the turbidity of shampoo-treated and control fields during the same period (90 and 76 FTU, respectively); however, this turbidity difference was unrelated to pH and physicochemical characteristics of the insecticide.

Heavy rainfall was recorded on May 20th (122 mm) and September 4th–5th (161 mm) after typhoons moved through the area, causing considerable runoff from the fields. More rainfall was also recorded on June 1st, 7th and 11th, but these events did not produce any observed runoff, nor did a few summer storms in July and August.

Duplicate samples of water (500 mL) and surface soil (2–3 cm, approximately 250 cm^3) from imidacloprid fields were taken 2 h after transplanting of seedlings, and then at 4, 7, 13, 27, 55, 90 and 118 days. These samples were analysed for residues of this insecticide in the treated fields throughout the study period. To avoid photolysis following sampling, water samples were collected using amber glass bottles and stored in the dark at 4 °C, whereas soil samples were sealed in aluminum cans and stored in a freezer (−20 °C) until analysis.

For chemical analysis of imidacloprid residues in water and soil, the method of Baskaran et al. (1997) was followed. Samples were extracted using SPE cartridges (Varian Bond Elut C_{18}) that had been preconditioned with 5 mL of methanol and washed with an equal volume of dionised water. Water samples (100 mL) were not filtered but aspirated directly through the C_{18} column, and the eluate discarded. After drying, the columns were eluted with 2 mL methanol, which was subsequently evaporated under a gentle stream of N_2, and then redissolved in 1 or 0.5 mL of acetonitrile–water (20:80, v/v) for HPLC analysis. Soil samples were defrosted and let settle overnight in order to discard the top layer of excess water. About 30 g of soil (40 g of wet sludge, 20 ± 2% moisture) were extracted with 40 mL of acetonitrile–water (80:20, v/v) while being stirred on an end-over-end shaker for 2 h. After mixing, the sludge was centrifuged at

1200 × *g* for 20 min, and the aqueous supernatant filtered through a 0.2 μm glass-fibre filter (Millipore) into a flask. This process was repeated again with the remaining soil, and the combined extracts were concentrated using a rotary evaporator at 45 °C. Five milliliters of acetonitrile were added to the residual aqueous suspension, and the mixture was aspirated through a C_{18} column in the same way as the aqueous samples. Analytical recoveries were in the range 92–108% and 83–94% for water and soil samples, respectively.

All water and soil samples were analysed by high-performance liquid chromatography on a Shimadzu LC-9A equipped with a UV–VIS spectrophotometric detector, using an Inertsil ODS-3 column (15 cm × 4.6 mm i.d., 5 μm particle size, GL Sciences Inc.) kept at 25 °C, and mobile phase of acetonitrile–water (20:80, v/v) at a flow-rate of 1.5 mL/min. Imidacloprid was detected at 270 nm. Under these conditions, the calculated detection limit for 50 μL of sample injection was 0.02 μg/L (ppb) in water and 0.2 ng/g (ppb) in soil.

On transplanting day (May 14), 10 adult male and 10 female medaka fish were released in each field and allowed to reproduce. Spawning must have occurred very soon after the release, as newly born fry were abundant in all fields in just 2 weeks. Medaka eggs take about 1 week to hatch at the water temperature measured in the fields during that period (22–24 °C).

Adult fish were counted in each field after 4 and 7 days, and once a week thereafter for a period of a month, but rapid growth of the rice plants and algae rendered this census inaccurate after the first month. Under these conditions, population growth parameters were deemed unreliable and mortality rates were difficult to assess with reasonable accuracy.

Commencing on June 17 and once every subsequent month, 10 medaka fry were sampled randomly from each field, and taken to the laboratory for examination of sublethal effects. Endpoints included morphological abnormalities and indicators of physiological stress such as the ratio between body weight and length (Giesy et al., 2003), and parasite infestation.

Differences in effects between treatments were tested using one-way ANOVA on the log-transformed data set (body weight and length) or arcsine-transformed set (survival%) to adjust for normality. Tukey's test was used to compare treatment means among multiple groups whenever significant differences ($P < 0.05$) were found with ANOVA. The significance of the rate of malformation in Zpt-treated fields was determined by a chi-square test with reference to the rate in control fields.

3. Results and discussion

3.1. Adult medaka survival

Given the nominal concentrations of Zpt at the time of each shampoo application (185–370 μg/L), and since the calculated LC_{50} to this fish species is between 60 and 120 μg/L for 24–72 h exposure (M.G.A. Sarker, personal communication), more than half of the medaka would be expected to die before the second shampoo application took place 3 days later. However, only nine medaka (23%) died in the Zpt-shampoo paddies within the first 4 days, and one more in the second week. During the same period, two medaka (5%) died in imidacloprid fields, when concentrations of this chemical were >30 μg/L (Table 2), whereas no deaths were recorded in the controls (Fig. 1). Further losses of fish in subsequent weeks could not be attributed to the acute toxicity of these chemicals alone because the same occurred in the control fields. Evidence of black-crowned night herons (*Nycticorax nycticorax*) visiting the experimental fields at night may have also played a role in the rapid disappearance of adult medaka in the ensuing weeks. Thus, after a month adult medaka losses reached proportions of 38, 35 and 15% in the imidacloprid, Zpt-shampoo and control fields, respectively.

Table 2
Concentrations of imidacloprid in water (μg/L) and soil (μg/kg dry weight) from the paddy fields

Days after planting	Water	Soil
0.1	239.2 (220.9)	–
4	32.9 (23.5)	24.3 (3.4)
7	5.2 (2.8)	5.5 (0.6)
13	2.4 (1.5)	13.2 (5.5)
27	1.1 (0.3)	14.8 (13.4)
55	0.6 (0.2)	7.1 (0.1)
90	0.1 (–)	24.7 (17.0)
118	1.1 (2.1)	1.6 (3.2)

Figures in brackets indicate 95% confidence limits. The day before planting, rice seedlings had been treated with Admire GR insecticide (1% imidacloprid) at 215 g a.i ha^{-1}.

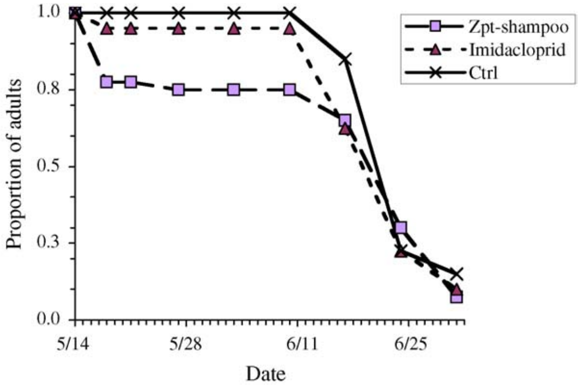

Fig. 1. Survival rate of adult medaka fish in the experimental paddies. Only differences between Zpt-treated and other fields were significant ($P < 0.01$, ANOVA) during the first month.

The fact that abundant newly hatched fry were present in the shampoo fields, with their numbers being similar among all fields, and no deaths were observed in the subsequent months in spite of the repeated doses of Zpt-shampoo every 3–4 days, indicate that effective concentrations of Zpt were well below its lethal level for 24-h exposure.

3.2. Malformations

Very few medaka fry collected from Zpt-shampoo fields on June 17 and from a control field on September 16 had abnormal development of their tails (2 and 1, respectively, Fig. 2a and b). No other signs of malformation, such as torsion of spinal cords, were found among the 240 specimens examined. Although the rate of malformation was slightly higher in the Zpt-shampoo fields ($2.5 \pm 1.3\%$) than in the control fields ($1.3 \pm 0.6\%$), such difference is not statistically significant ($P > 0.25$, chi-square test). No malformations were observed in medaka fry from imidacloprid-treated fields.

The overall rate of malformation was, therefore, low and fell within the natural background found in oceans and rivers (Klumpp and van Westernhagen, 1995; Luckenbach et al., 2001). Furthermore, malformations among medaka from the Zpt-treated fields were only observed during the first month, suggesting that no bioaccumulation of Zpt occurred despite the continuous addition of Zpt-shampoo throughout the experimental period (4 months).

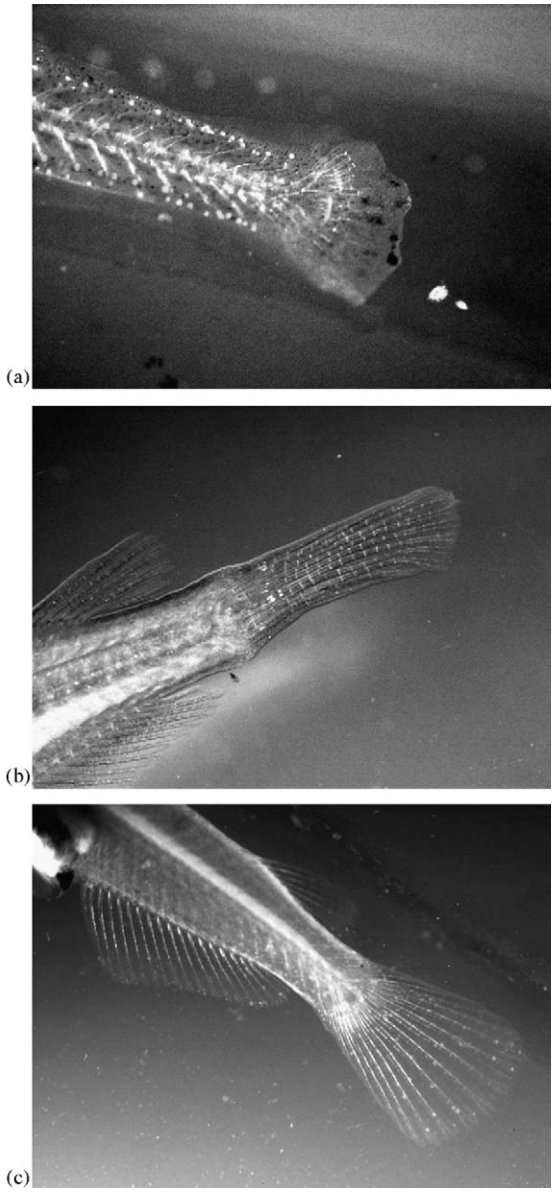

Fig. 2. Abnormal development of tails in medaka fry from Zpt-shampoo fields (a) and control fields (b), compared to a normal specimen (c).

Although no measurements were made on the concentrations of Zpt, its amount in the paddy system (water and soil) fluctuated somewhere between the above nominal value and nearly zero before a new input was made 3–4 days later. Because of the unstable nature of this chemical (Thomas, 1999) and its quick degrada-

Table 3
Proportion of medaka infested by the parasite *Trichodina domerguei* (figures indicate percentages, with the number of fish examined in brackets)

	Zpt-shampoo	Imidacloprid	Control	Total
(a) Mild	1.3 (1)	48.8 (39)	23.8 (19)	24.6 (59)
(b) Severe	– (0)	23.8 (19)	– (0)	7.9 (19)
Total infested	1.3 (1)	72.5 (58)	23.8 (19)	32.5 (78)
Healthy	98.8 (79)	27.5 (22)	76.3 (61)	67.5 (162)
Total	100 (80)	100 (80)	100 (80)	100 (240)

tion under sunlight, it became apparent that actual Zpt exposure to medaka eggs and embryos in the field was reduced enormously within a few hours. With the half-life of Zpt ranging from 30 min in anaerobic sediments to 51 min in water from ponds (Turley et al., 2000) such as those in flooded paddies, a simple calculation shows that less than 1% of the initial Zpt amount remains in water after 6 h. Moreover, using a biphasic dissipation model as described by Turley et al. (2000), the active ingredient of shampoo will reach undetectable concentrations (<0.02 μg/L) in the system between 1 and 2 days.

These field results fit well with the predicted low exposure based on the known chemical features of Zpt (Table 1), and reinforce the proposition that physico-chemical processes—mainly dissipation and sorption are essential to understand the real exposure levels of toxicants (Sánchez-Bayo, 2004), and consequently the biological effects that occur in environmental situations.

3.3. Other sublethal effects

Parasites were found on the first monthly sampling: all medaka fry from imidacloprid fields were infested with *Cychlochaeta* (=*Trichodina*) *domerguei*, a protozoan ectoparasite commonly found in freshwater fish. This parasite was also present in medaka from the control fields in lower proportions (40%), but was absent in all but one fry from the Zpt-shampoo field (Table 3). The degree of infestation varied among individuals, with most fry having the fins and tails covered with *Trichodina*, a condition described here as 'mild' (Fig. 3a), and fewer fish covered by a dense matt of such parasites in cases of 'severe' infestation (Fig. 3b).

Trichodina infestation levels fluctuated but remained high (40–100%) in imidacloprid fields for the entire experimental period, following the same temporal trend as in control fields (Fig. 4). However, levels in the control (0–45%) were significantly lower than those in imidacloprid fields, and never reached the 'severe' condition found exclusively in the latter treatment.

It is well established that fish exposed to pollutants develop a stress syndrome characterized by increasing anaerobic metabolism, which initially results in an elevated lactate concentration in blood followed by hyperglycemia (Sancho et al., 1997). Protein catabolism is then required to compensate for energy losses (Pfeifer and Weber, 1979). The reason for this metabolic change

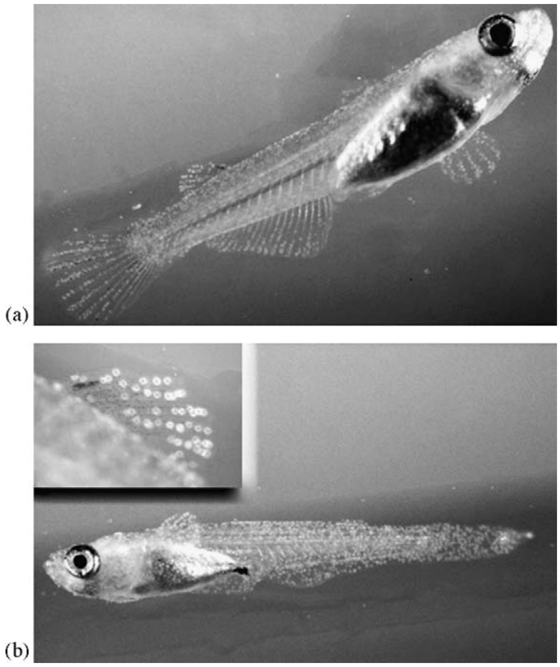

Fig. 3. Infestation of medaka fry by the parasite *Cyclochaeta* (=*Trichodina*) *domerguei* (white circles in 'b' insert). (a) 'Mild' condition affecting fins and tails, (b) 'severe' infestation of entire body and fins.

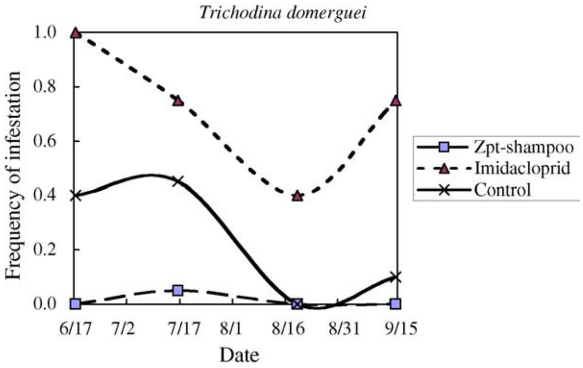

Fig. 4. Temporal fluctuation of medaka fish infested by *Trichodina domerguei* parasites in treated and control fields throughout rice cultivation. Each data point represents 20 fish.

seems to be related to gill damage, which reduces the amount of oxygen taken up by fish (Evans, 1987). Evidence of physiological stress in juvenile medaka from both treated fields was found after measuring their body weight and length. The ratio between these two parameters (W/L), which has been used as a health indicator or biomarker (van Gestel and van Brummelen, 1996), decreases in value whenever fish undergo physiological stress. To eliminate other confounding factors such as parasites, statistical analyses were performed on three separate groups (Fig. 5). Among the healthy group, medaka from Zpt-shampoo fields showed significantly lower W/L ratios than those from other fields ($P < 0.001$), whereas all fish suffering 'severe' infestation had the lowest W/L ratios. In addition, the most stressed medaka lived in the Zpt-shampoo fields, followed by those in imidacloprid fields, both of which differed significantly from fish in the controls ($P < 0.01$).

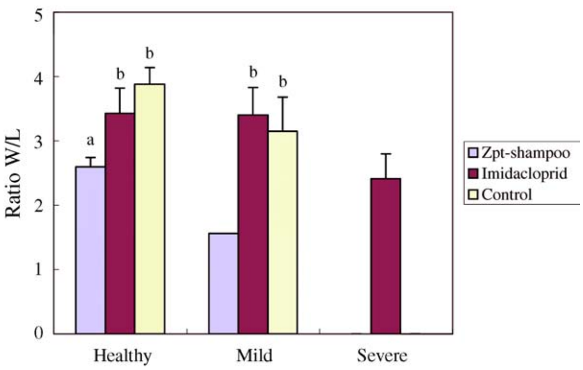

Fig. 5. Stress related ratio weight/length (W/L) among healthy medaka from treated and control rice fields and fish affected by 'mild' or 'severe' *Trichodina* infestation. Significant differences were found between Zpt-shampoo and control (a, $P < 0.001$) in the healthy fish group. Bars indicate standard error of the mean.

The stress effect was proportional to the intensity of exposure, reflecting the constant, chronic input of Zpt-shampoo compared to a single application of imidacloprid, as the effectiveness of the latter chemical was supposed to decrease in time. In fact, analysis of paddy water showed imidacloprid concentrations varying from 0.24 mg/L at 2 h after transplanting to 33 and 1.1 μg/L at 4 and 27 days, respectively (Table 2). The calculated half-life in water during the first month is 4 days ($r^2 = 0.74$), though some imidacloprid was lost in the runoff due to a typhoon that hit the area in the first week of exposure. Residues in water decreased in the subsequent months and were maintained at 0.75 μg/L in equilibrium with those in the soil (13 ± 9 μg/kg dry weight) until the end of the experiment. Consequently, only during the initial week were adult medaka and their eggs exposed to considerable concentrations (33–240 μg/L) of this insecticide, whereas their offspring were less affected afterwards.

Stressed fish from either imidacloprid or control fields suffered from *Trichodina* infestation, which is natural, due to the lower immunity associated with this condition (Schwaiger et al., 1997; Mitchell, 2001). But the lack of parasite infestation on stressed medaka fry in the Zpt-shampoo fields is most remarkable. To explain this inconsistency, one must recall that Zpt is a potent biocide with cytotoxic and bactericidal activity (Dinning et al., 1998), and its disinfectant properties are responsible for the direct elimination of *Trichodina*. It is also likely that Zpt may have eliminated other protozoans and soil microorganisms, many of which are beneficial to the functioning ecology of paddy fields (Suyama et al., 2001), a point recently addressed by Petersen et al. (2004).

4. Conclusions

Effects of zinc pyrithione, a biocide used in antidandruff shampoos and antifouling paints, and the agricultural insecticide imidacloprid, on medaka fish were studied under rice field conditions. Exposure of medaka to imidacloprid had no significant effect on its survival/mortality rate, whereas Zpt eliminated one-fourth of the fish population in the first 2 weeks, quite fewer than expected by the LC_{50} of the nominal concentration of Zpt present.

Although previous laboratory studies (Goka, 1999) showed that zinc pyrithione causes backbone deformities in medaka embryos, rates of malformation in fields treated periodically with anti-dandruff shampoo were within the natural background. These results indicate that environmental consequences of this biocide may be offset by factors such as photolysis and anaerobic degradation in sediments (Maraldo and Dahllo, 2004). The unexpected absence of malformations in the Zpt-shampoo fields demonstrates that the actual exposure of fish in the paddy was undoubtedly reduced to levels below the teratogenic threshold of Zpt in a few hours, due to the fast dissipation properties of this biocide (Turley et al., 2000). By contrast, laboratory toxicity tests use constant levels of chemicals for 24 or 48 h and cannot be used to predict the real effects that take place in the environment.

No malformations, however, were observed in fields treated with imidacloprid, but both chemicals caused physiological stress in juvenile medaka, with weaker fish suffering a massive infestation by *Trichodina* ectoparasites in imidacloprid fields but not in those treated with Zpt-shampoo, supposedly due to the disinfectant action of zinc pyrithione.

Acknowledgements

The advice of Dr. Shosaku Kashiwada and his help in determining deformities in medaka are very much appreciated. We thank Yutaka Ogamino for identifying the parasites, and Yoshio Suzuki and staff at the Kawakami F.C. Experimental Field Station (Chizuko Yoshida, Daisuke Yamashita and Teruko Okubo), for planting the rice, providing meteorological data and looking after the paddy during the experiment. Comments by an anonymous reviewer helped improve the original manuscript.

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