Chapter 6

The Ecotoxicity of Pyrimethanil for Aquatic Biota

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Additional information is available at the end of the chapter

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Abstract

Via the application of agrochemicals, farmers currently guarantee high productivity of fruit and vegetable crops. However, pest reduction using excessive amounts of such chemicals has a negative effect on aquatic organisms. The spray-drift, leaching, run-off or accidental spills occurring during or after application has become a serious and increasing problem for aquatic ecosystems. Pyrimethanil (PYR) is one of the most used fungicides. Such increase has heightened the interest in studying the potential risk and influence of PYR on the environment. In this chapter information on the PYR environmental risks for aquatic organisms was divided into three different approaches: (i) assessment of toxic effects of the pure active ingredient or the commercial formulation on primary producers, (ii) assessment of toxic effects of the pure active ingredient and PYR formulation on aquatic animals, and (iii) estimation of the role of PYR as an environmental disturber by triggering avoidance response. The available data provide evidences that PYR is potentially toxic for many aquatic species, affecting survival, reproduction, feeding, growth, and that it can disturb the environmental quality with no direct effect at the individual level by inducing organisms to migrate to less impacted areas.

Keywords: aquatic organisms, ecotoxicity, environmental disturbance, fungicide, pyrimethanil

1. Introduction

Increasing food requirements exert a constant pressure for intensifying agricultural activities, recognized, nowadays, as one of the most important economic activities in many high and low income countries [1]. In fact, agriculture has been considered a feasible solution for reducing the levels of poverty and hunger given that the vast majority of poor people in developing countries are concentrated in rural areas [2]. The high demand for agricultural products requires optimizing the production to reduce the loss due to crop diseases such as those caused by fungi. Although incentives for agriculture optimization and development are usually paralleled by sustainable practices, intensive agricultural practices and the pursuit for more profitable productions have unfortunately escalated the increase in the use of agrochemicals against crop pests/pathogens. Additionally, the agrochemical market represents an important economic sector for many countries [3]. According to the previously mentioned authors, although the use of chemicals such as lime sulfur and Bordeaux mixture as fungicides began in the mid-1800s, only in the 1960s did fungicides with specific (systemic) modes of action become protagonists in controlling against fungal pathogens. The more serious consequence is the fact that the impact of agrochemicals is not only on pests and pathogens, but also on non-target organisms inhabiting adjacent areas, including humans. The excessive and indiscriminate application of agrochemicals linked to a lack of legal control about their use, commercialization and regularization in many countries has given agrochemicals a primary role of concern in environmental management. Among the groups of chemicals used in agriculture against pathogens, fungicides are the third most used agrochemical group, representing ca. 23% of sales on the agrochemical market [4]. Contrary to most agrochemicals, fungicides are frequently applied in a prophylactic manner several times per season, although at lower application rates than most herbicides and insecticides, which increases the risk of chronic exposure to aquatic biota [5]. On the other hand, some organisms can develop resistance to fungicides after relatively short periods (years) of exposure, resulting in fungal pathogens being responsible for important economic losses of fruit and vegetable products [5, 6].

Via the application of agrochemicals, farmers currently guarantee high productivity of fruit and vegetable crops. However, reduction of crop losses by using excessive amounts of such chemicals has a negative effect on aquatic organisms. The spray-drift, leaching, run-off or accidental spills occurring during or after application of agrochemicals has become a serious and increasing worldwide problem for aquatic ecosystems [7, 8]. Pyrimethanil (PYR) is one of the most used fungicides that has been detected in many aquatic ecosystems [5] and one of the most frequently used in European vineyards [9, 10, 11]. Such increase has heightened the interest in studying the potential risk and influence of PYR on the environment [3, 5, 12–14].

The main objective of this chapter is to provide information on the environmental risks posed by PYR for aquatic organisms. For this, PYR chemical characteristics as well as its potential risk for the aquatic environment will firstly be provided and subsequently three different approaches will be discussed: (i) assessment of toxic effects of the pure active ingredient or the commercial formulation on primary producers using traditional assays with forced exposure,

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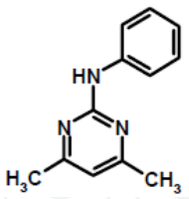
(ii) assessment of toxic effects of the pure active ingredient and PYR formulation on aquatic animals using traditional assays with forced exposure and *in situ* experiments, and (iii) estimation of the role of PYR as an environmental disturber by triggering avoidance response in a non-forced exposure system.

2. Pyrimethanil: Characteristics and hazard potential

The fungicide PYR (*N*-(4, 6-dimethylpyrimidin-2-yl)-aniline; CAS number 53112-28-0) is an anilinopyrimidine fungicide that inhibits the secretion of fungal enzymes produced in the infection process [15, 16]. It was recently developed to act on resistant fungi strains, mainly to control *Botrytis cinerea* in grapes (wine), *Venturia inaequalis* in apples and *Botrytis* spp. in protein peas [17], reason for which its use has increased greatly [18, 19]. PYR rapidly penetrates the cuticle and inhibits the secretion of fungal enzymes required for the infection process, blocking the ability of fungi to degrade and digest the plant tissues, and thus stopping development of the disease [17, FAO, see http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/ JMPR/Evaluation07/Pyrimethanil.pdf]. The commercial products that contain PYR as active ingredient are Clarinet®, Mythos®, Rubin®, Scala®, Siganex®, Vision®, and Walabi®, which are currently used both pre- and post-harvest to protect various crops such as apple, banana, carrot, citrus, grape, melon, onion, potato, strawberry, and tomato [16, 18, 20, see also www.bayercropscience.com.br/Site/nossosprodutos/protecaodecultivosebiotecnologia/DetalheDoProduto.fss?Produto=44]. According to the EFSA report [17], PYR is rapidly excreted once orally absorbed, it has no potential to bioaccumulate, has a low acute toxicity, is not teratogenic and seems to have no neurotoxic effect; however, studies have observed acute and chronic toxicity for non-target organisms [7, 14, 21–27] that converts PYR into an environmental disturber of concern. Unfortunately, despite the intensive agricultural use of PYR, there is not an exhaustive study regarding the effects on adjacent aquatic ecosystems. This is possibly related to the assumption that PYR has a short half-life, with fast degradation [17, 28] and, therefore, possible toxic effects may occur at short term but are minimized at mid- and long-term. Some chemical and (eco)toxicological characteristics of PYR published by EFSA [17] can be seen in Table 1.

The regulatory decision to approve a given agrochemical should be based not only on its efficiency in controlling the pest/pathogen, but also on the potential environmental impact on non-target organisms inhabiting both target and nearby areas. Given the lack of information on PYR biological effects and the imminent need to expand the range of toxicity tests, a series of recent studies have been conducted to fill in this information gap. This chapter is to present these results and integrate them with the information that was already available. With the latter purpose, different aquatic organisms from different levels of biological organization have been used in ecotoxicological studies in the past years to evaluate the potential risks due to exposure to PYR via different routes. Beside traditional toxicity tests, approaches taking into account multigeneration responses, temperature influence and behavioral endpoints as well as *in situ* exposures have been performed and will be discussed in the next sections.

Chemical name (IUPAC)		*N*-(4, 6-dimethylpyrimidin-2-yl) aniline
Chemical name (CA)		4, 6-dimethyl-*N*-phenyl-2-pyrimidinamine
Molecular formula		$C_{12}H_{13}N_3$
Molecular mass		199.28 g mol^{-1}
Structural formula		
Temperature of decomposition		189.54 to 344.74 °C
Flammability		Not flammable
Explosive properties		Not explosive
Skin irritation		Not irritating
Eye irritation		Not irritating
Genotoxicity		No evidence
Degradation time in water and sediment	DT_{50} water	8.9 to 24 days
	DT_{90} water	70 to 99 days
	DT_{50} whole system	40 to 121 days
	DT_{90} whole system	Not stated and 134 days
Toxicity for aquatic organisms	Rainbow trout	LC_{50} (96 h): 10.56 mg L^{-1}
	Daphnia sp.	EC_{50} (96 h): 2.9 mg L^{-1}
	Green alga*	EbC_{50}/ ErC_{50} (96 h): 1.2/5.84 mg L^{-1}
	Daphnia magna	NOEC (reproduction, 21 d): 0.94 mg L^{-1}
	Chironomus riparius	NOEC (emergence, 28 d): 4.0 mg L^{-1}
Ecotoxicological data		Harmful

CA: Chemical Abstract; DT_{50} and DT_{90}: period required for 50% and 90% dissipation; EC_{50}: median effective concentration; EbC_{50}: the concentration at which 50% reduction of biomass is observed, ErC_{50}: the concentration at which 50% reduction of growth rate is observed, IUPAC: International Union of Pure and Applied Chemistry; LC_{50}: median lethal concentration; NOEC: no observed effect concentration. * Species name is not provided.

Table 1. Chemical and (eco)toxicological characteristics of pyrimethanil [17].

3. Toxicity of pyrimethanil to primary producers (microalgae and macrophytes)

Agrochemicals can considerably affect the structure of algal communities generating functional changes, due to alterations in biotic interactions. Freshwater macrophytes and microalgae usually are not the target of agrochemicals; however, the potential impact that these compounds can have on primary producers is well known [29]. Various studies alerting to the risk of excessive excessive pesticide application and consequent pollution of aquatic environments have been performed using different microalgae, duckweeds, and the aquatic plant *Myriophyllum aquaticum* as test organisms [8, 30–36].

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The growth of the floating plants *Lemna minor* and *L. gibba* was inhibited by pure PYR with an IrC_{50} of 23 and 7.8 mg L^{-1}, respectively [23, 28]. In the same concentration range, the growth of the unicellular green algae *Scenedesmus acutus*, *S. obliquus*, *Desmodesmus subspicatus*, and *Raphidocelis subcapitata* (=*Pseudokirchneriella subcapitata* and also *Selenastrum capricornutum*) was affected [7, 23, 25, 28]. On the other hand, the diatom species *Gomphonema parviculum* revealed lower PYR effect concentrations (IrC_{50} = 0.24 mg L^{-1}, unpublished data). After 4-days-exposure to 0.2 mg L^{-1} PYR, maximal photosynthetic capacity of the macrophytes *Callitriche palustris* and *Elodea canadensis* was significantly inhibited [21].

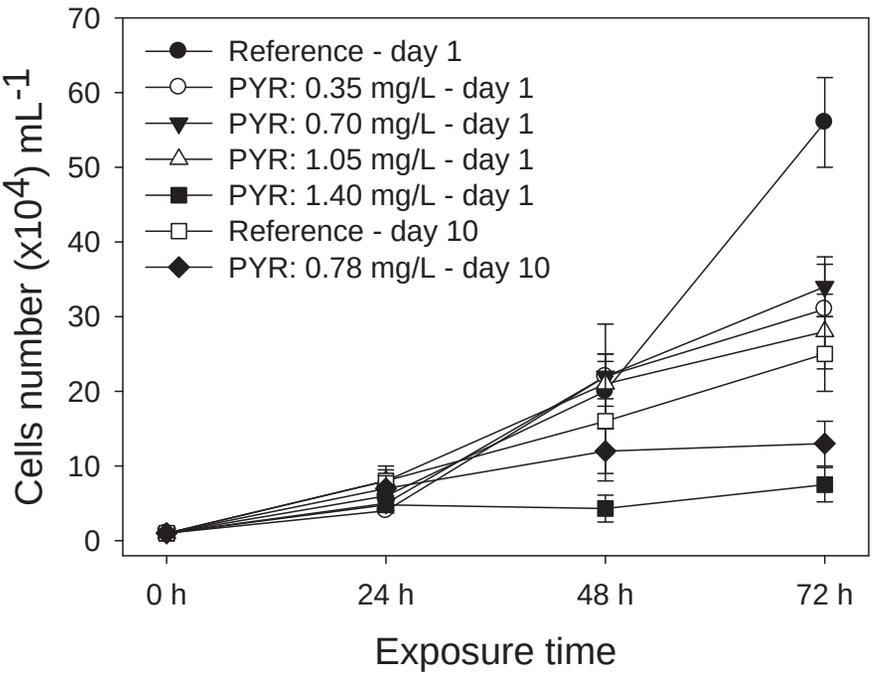

Figure 1. Growth curves of the microalgae *P. subcapitata* after 72 h exposure to reference and pyrimethanil-treated mesocosm samples taken at days 1 and 10 post-application.

Also, toxicity tests with water from PYR-treated mesocosms (commercial formulation Mythos®; initial PYR concentration of 1.4 mg L^{-1}) were performed with the microalgae *P. subcapitata* by Shinn et al. [14]. In the latter study, water samples were taken at day 1 and 10 after PYR application to verify whether immediate effects and those after subsequent PYR dissipation, respectively, would be different. The cell growth during 72 h exposure to 1-d PYR-treated mesocosm water diluted at different concentrations and to the 10-d undiluted PYR-treated mesocosm sample is summarized in Figure 1, together with the growth of cells exposed to water from the reference mesocosm. *P. subcapitata* cells exposed to samples from PYR-treated mesocosms taken at day 1 had their growth strongly inhibited, whereas the effects on cell growth were less pronounced at day 10, when PYR concentrations showed a reduction of 45% (0.78 mg L^{-1}) in relation to day 1 (1.40 mg L^{-1}). According to Shinn et al. [14], these results indicate that depending on the concentration at which PYR reaches the affected aquatic system,

immediate effects on phytoplankton species can be observed. However, assuming an interruption of the contamination by the fungicide, these same effects may become considerably attenuated in view of the dissipation of PYR from the aquatic compartment.

4. Toxicity of pyrimethanil to aquatic animals

The toxic effects of pure PYR on different aquatic animals and responses have also been addressed. For the cladocera *Daphnia magna* the 96 h LC_{50} (lethal concentration to 50% of exposed organisms) of pure PYR ranged from 1.2 to 2.9 mg L^{-1} and the no observed effect concentration (NOEC) on reproduction after 21 d exposure from 0.5 to 0.9 mg L^{-1} [17, 23]. Surprisingly, *D. magna* exposed to 1.0 mg L^{-1} PYR (LOEC for reproduction, 21 d) at 14, 16, and 19 °C did not produce a F_1-generation [23]. The EC_{50} for the reproduction of the sister species *D. pulex* was 0.69 mg L^{-1} and the NOEC was 0.015 mg L^{-1} [25].

Regarding aquatic insects, the NOEC of pure PYR for the non-biting midge *Chironomus riparius* was 4 mg L^{-1} [22] and the EC_{50} for the phantom midge *Chaoborus flavicans* was 1.78 mg L^{-1} [25]. Within a similar PYR range, the oligochaete *Lumbriculus variegatus* revealed a NOEC of 4 mg L^{-1} with respect to reproduction [23] and the snail *Physella acuta* presented embryo LC_{50} of 0.402 mg L^{-1} [24]. The lethal PYR concentration for 50% of a rainbow trout population (*Oncorhynchus mykiss*) was 14 mg L^{-1}, whereas the NOEC for the parameter dry weight was 0.07 mg L^{-1} PYR [37]. Survival and oxidative stress of the aquatic worm *Tubifex tubifex* has also been used as an endpoint to assess the toxicity of PYR [12]. Compared with results previously described, LC_{50} values were relatively high, between 39 and 49 mg L^{-1}, after 7 and 1 day exposures respectively. On the other hand, effects on the activity of catalase (increased activity) and glutathione-*S*-transferase (decreased activity) were observed at lower (25 mg L^{-1}) concentrations [12]. These same authors detected a quick (after 4 d) bioaccumulation of PYR in the worm, but that was reduced in the subsequent days.

Recently, impairment in the feeding ability of the tropical cladoceran *Ceriodaphnia silvestrii* was verified by Araújo and collaborators (unpublished data) using the post-exposure feeding endpoint, increasingly used in ecotoxicological studies [38–41]. In the study of Araujo and collaborators (unpublished data), a PYR contamination scenario was simulated by applying the commercial formulation Mythos® to a mesocosm system. Two treatments were considered: reference (non-contaminated) mesocosms and mesocosms contaminated with PYR at 1.40 mg L^{-1}. The *in situ* exposure started one day after application, when *C. silvestri* individuals (3rd brood; 3 days old) were exposed for 24 h in the mesocosms in 250 mL cylindrical chambers as shown in Figure 2. After this period organisms were removed, transported to the laboratory in containers with the respective mesocosm water, checked for mortality, and surviving *C. silvestri*were fed with a *P. subcapitata* algal suspension of known concentration for 4 h, time after which remaining cells were recorded.The feeding of individuals exposed to the commercial formulation Mythos® applied to mesocosm systems was inhibited up to 31 ± 12%, with no lethal effect. The absence of an acute effect was expected as according to published data the lethal toxicity of PYR for the crustacean *D. magna* is known to occur at concentrations around

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3 mg L^{-1} [37] and chronic toxicity for *D. magna* reproduction at approximately 1 mg L^{-1} [23]. The PYR effects on the feeding of *C. silvestri* showed post-exposure feeding can be a suitable endpoint to discriminate the effects of contamination caused by the fungicide PYR. Since feeding is a mechanism by which organisms obtain energy for many biological functions (e.g., development, growth, reproduction, survival), the impairment of the feeding capacity may be critical not only for the organisms themselves (reduced competitive ability, energy imbalance, higher susceptibility to predation), but also for ecosystem functioning as an unbalance in the food web (increase in phytoplankton community due to a decrease in grazing) may have consequences at the community level [39].

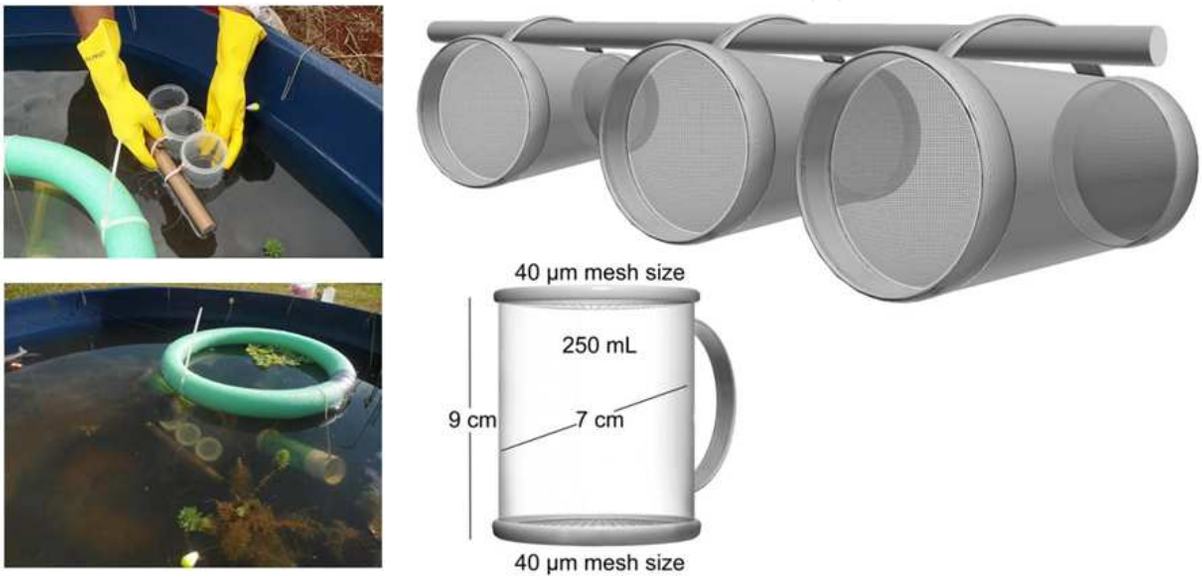

Figure 2. Exposure of *Ceriodaphnia silvestri* to pyrimethanil in mesocosms and schematic diagram of the exposure chamber.

Aiming to assess the suitability of the yeast *Saccharomyces cerevisiae* gene expression–based assay for screening the toxicity of worst-cases of soil and water PYR contamination, soils were sprayed with the commercial formulation Scala® to simulate accidental spill doses and runoff events [42]. The authors found that, although less sensitive, the yeast-based assay correlated well with the toxicity of runoff and soil samples to the following aquatic and soil organisms: *D. magna* (48 h-survival, LC_{50} of 0.8 mg L^{-1}, and 21 days-reproduction, EC_{50} of 0.49 mg L^{-1}), *C. riparius* (10 days growth, EC_{50}: 92.5 mg L^{-1}), the soil invertebrates *Folsomia candida* (28 days reproduction, EC_{50}: 19.9 mg kg^{-1}) and *Enchytraeus crypticus* (28 days reproduction, E_{50}: 30.3 mg kg^{-1}), and the nematode *Caenorhabditis elegans* (72 h-reproduction, EC_{20}: around 1.4 mg L^{-1}).

A multi-parameter approach to assess the toxicity of PYR in a probable global change scenario has been used by Müller et al. [22], Seeland et al. [23] and Scherer et al. [25], based on the assumption that under climate change conditions warmer and more humid environments are expected, leading to conditions suitable for fungi development, thus an increase in the use of fungicide [22]. Therefore, these authors evaluated if the toxicity of PYR for invertebrate species (*C. riparius*, *D. magna, D. pulex*, *P. acuta*) alters with increasing temperature. Lethal PYR toxicity

to *C. riparius* increased when combined with increasing temperature [23]. The loss of genetic diversity in *C. riparius* cohorts when exposed to PYR (2 mg L^{-1} = NOEC/2 of PYR for reproduction) for multiple generations also depended on the thermal regime; genetic diversity became reduced by approximately 20% under thermal simulation of a typical cold or warm year in 1990–2005 and by 42% under a suboptimal temperature regime expected for a warm year in Europe in 2050–2080 under climate change conditions [22]. Likewise at suboptimal temperature conditions, the thermophil snail *P. acuta* presented higher susceptibility to toxic effects of PYR [24]. However, other studies indicate that PYR toxicity is highest at current optimal temperature regimes. The release of neonates from adult *D. magna* exposed to 0.5 mg L^{-1} (NOEC of PYR for reproduction) for multiple generations under dynamic temperature scenarios was most affected by PYR at a favorable temperature range (20 to 27 °C) [23]. In *D. pulex*, the inhibition of reproduction was not observed at suboptimal 15 °C, but at optimal 20 °C and 25 °C [25]. Interestingly, those PYR effects vanished in presence of kairomones from the predator *Chaoborus flavicans*.

5. Role of pyrimethanil as environmental disturber: Avoidance assays

It has been hypothesized that contaminants can act as toxicants as well as habitat disruptors. The former role is characterized by directly measuring acute or chronic responses in organisms, while their role as habitat disruptor is directly linked to effects on habitats, reducing their quality and triggering avoidance before toxic effects are detected. The latter effect is particularly important given that concentrations at which it might occur could be considered non-risky as no toxic effect at the individual level would be usually observed [43, 44]. Habitat disturbance caused by contamination as a result of agricultural activities may, therefore, be considered an additional factor that increases the threat of local population decline [26, 45].

Given the above, a new approach based on avoidance as an endpoint and using a non-forced exposure system has been proposed to assess the role of contaminants as environmental disturbers. This approach considers that contaminants as environmental disturbers can change the community structure with no direct toxic effect on organisms as they may be able to detect and avoid contaminants [26, 43–45]. The exposure system used here creates a contamination gradient in which organisms can freely move across different levels of contamination and choose the less contaminated zone. A few studies have tested this methodology and proved that contamination levels lower than those considered potentially dangerous for organisms can trigger avoidance response by many aquatic organisms [26, 43, 44]. As a consequence, an ecosystem can suffer structural changes as individuals able to detect contamination move towards less contaminated zones [44–46].

Avoidance tests in non-forced exposure systems (Figure 3) in which a PYR gradient was simulated have been performed with fries of *Danio rerio* [26] and tadpoles of amphibians *Lithobates catesbeianus* and *Leptodactylus latrans* [27]. Data obtained from these experiments showed that the spatial distribution of the three species was influenced by the presence of PYR. Almost all organisms of all three species avoided PYR when the concentration was around 1

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mg L^{-1}. The results indicate, therefore, that organisms could react by avoiding a given environment before deleterious toxic effects set in and are detected via traditional acute endpoints. Based on the organisms' accumulated frequency along the system, the median preferred concentration, PC_{50} – concentration/dilution above or below which was preferred by 50% of organisms – and the PC_{25} and PC_{75} were calculated (Figure 3). For the three species, PC_{25}, PC_{50}, and PC_{75} were very similar and in general concentrations higher than 0.5 mg L^{-1} were avoided by 50% of the population of tadpoles and fries.

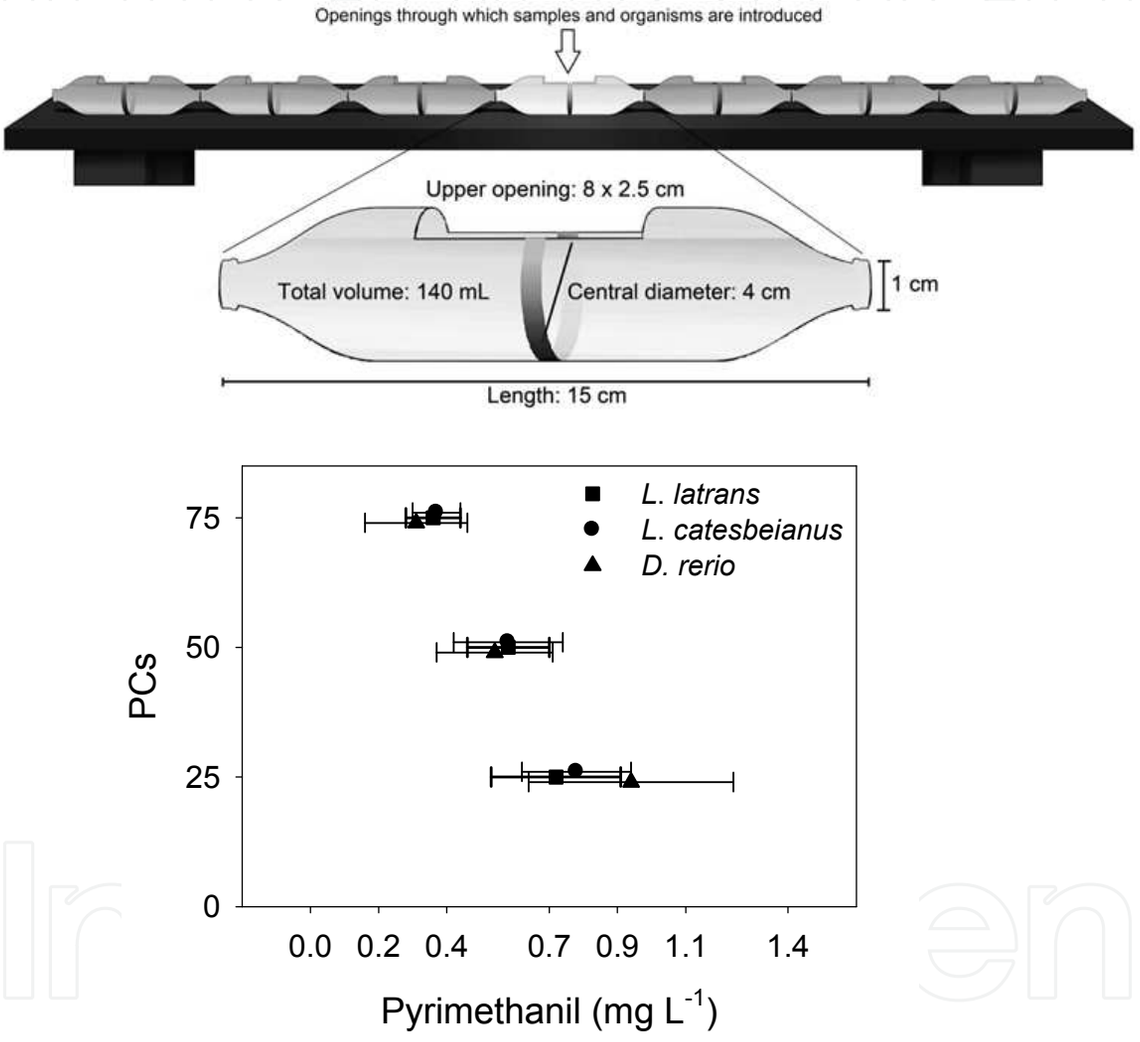

Figure 3. Schematic representation of the multi-compartmented non-forced system used for simulating a pyrimethanil gradient during avoidance assays (upper) and preferred concentration by 25, 50, and 75% (PC_{25}, PC_{50}, and PC_{75}) of tadpoles of two species of amphibians (*Leptodactylus latrans* and *Lithobates catesbeianus*) and of fries of *Danio rerio* exposed to a pyrimethanil gradient for 4 h (lower).

According to these findings, we emphasize the importance of taking into account the risk of the presence of plant protection products in the environment, even at non-lethal concentrations, due to their potential to trigger emigration. The presence of PYR can be a decisive factor in the habitat selection process of many species, such as shown in Figure 3. The disturbing

effect of contaminants on ecosystems can be comparable to the loss and fragmentation of habitats [47, 48]. Habitats with reduced quality due to presence of contaminants probably may support a smaller population as well as lose the capacity to serve as sink habitats for surrounding populations [48]. Since avoidance experiments can provide information about contamination-driven habitat selection, the use of non-forced exposure systems is therefore encouraged in environmental risk assessment with agrochemicals.

6. Final remarks

Undoubtedly, agrochemicals are potentially dangerous for aquatic organisms. The PYR concentration causing 50% reduced offspring in the most vulnerable aquatic species *D. pulex* (OECD model organism) is almost identical to the predicted environmental concentration of PYR in surface waters nearby apple orchards (0.089 mg L^{-1}, [17]). Thus, zooplankton communities may be at risk in case of expected PYR runoff into surface waters. If considering a risk safety factor of ten traditionally used for pesticides tested in chronic standardized bioassays with species from three trophic levels (algae, daphnids, fish), a PYR concentration of 0.9 mg L^{-1} should not induce adverse biological effects. Experiments with non-model species imply, however, that in particular Physidae are at risk at <0.9 mg L^{-1}. This result may recommend for the inclusion of different mollusk species in ecological risk assessment programs [24].

The similarly PYR-sensitive diatom *G. parviculum* [unpublished data] serves as important food source for grazers such as Physidae and one may therefore assume that indirect effects on the food web will appear in PYR-contaminated habitats in addition to the direct growth inhibition of the diatom, a result derived from single-species tests. Other indirect effects of 0.9 mg L^{-1} PYR may arise from the 50% avoidance behavior of the frogs *L. catesbeianus* and *L. latrans* observed after 12 h of exposure [27]. At a similar concentration range, the fish *D. rerio* avoids PYR contaminated freshwaters after 4 h (AC_{50} = 1.1 mg L^{-1}) [26]. The migration of top predators from certain habitats could however provide improved conditions for predated species (top-down effect), in particular for macroinvertebrates and algae being more PYR tolerant.

The available information provides evidences that PYR is potentially toxic for many aquatic species, affecting survival, reproduction, feeding, growth, and that it can disturb the environmental quality with no direct effect at the individual level by inducing organisms to migrate to less impacted areas. Although the amount of relevant information on the toxic potential of PYR on several species is increasing, little information is available on how the presence of PYR (and "inert compounds") can disturb broader environmental processes: chemical balance, direct effects on primary producers and consumers, changes in structure and functioning of the community and alterations in dispersion patterns. Further studies on the probable risk due to spray-drift, leaching, run-off, or accidental spills have to be encouraged. Presently, outdoor mesocosm studies taking into account different species, endpoints and exposure types in a more complex and relevant approach by using mesocosm experiments are ongoing. Given that the behavior and effects of PYR could vary between different climate conditions, the latter

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experiments are being performed across different climatic regions, from tropical to South- and North-temperate. Under these three environments, chemical dynamics of PYR in water and sediment are being followed for at least a 1 year period together with the monitoring of the complex local community, individual sub-lethal effects, changes in biodiversity and implications in ecological succession. The compilation of that information could help to understand the possible role that PYR plays environmental disturber for aquatic biota.

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