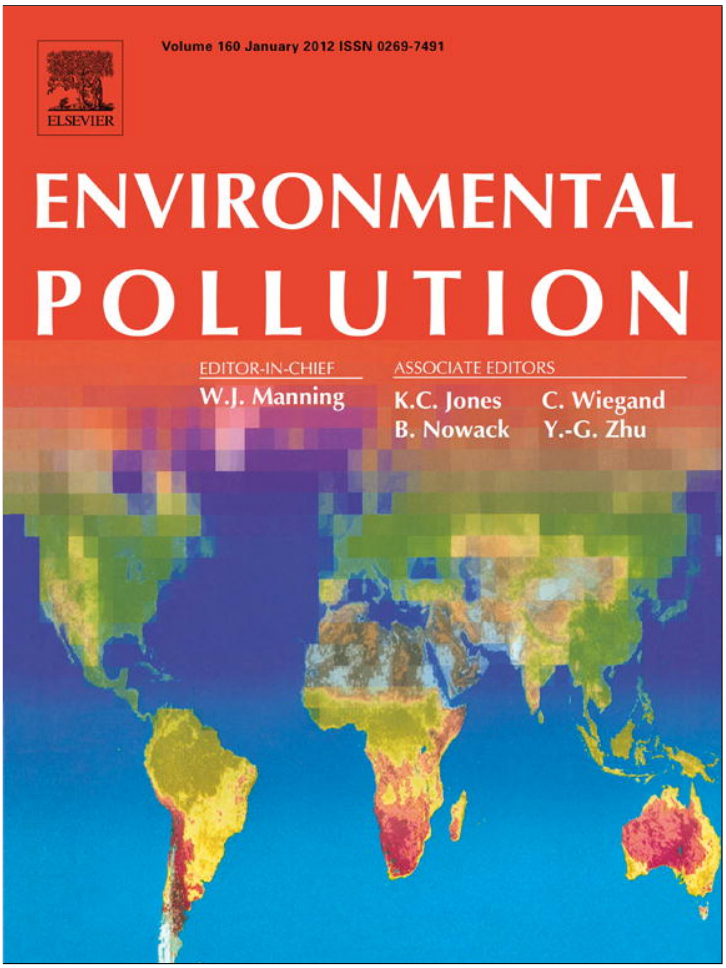

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Life stage-specific effects of the fungicide pyrimethanil and temperature on the snail *Physella acuta* (Draparnaud, 1805) disclose the pitfalls for the aquatic risk assessment under global climate change

Anne Seeland [a,b,*], Jennifer Albrand [a,b], Jörg Oehlmann [a,b], Ruth Müller [a,b]

[a] *Goethe University Frankfurt am Main, Department Aquatic Ecotoxicology, Max-von-Laue-Straße 13, D-60438 Frankfurt, Germany*
[b] *LOEWE Biodiversity and Climate Research Centre (BiK^F), Senckenberganlage 25, D-60325 Frankfurt, Germany*

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ABSTRACT

It can be suggested that the combined stress of pesticide pollution and suboptimal temperature influences the sensitivity of life stages of aquatic invertebrates differently.

The embryo, juvenile, half- and full-life-cycle toxicity tests performed with the snail *Physella acuta* at different concentrations (0.06–0.5 or 1.0 mg L^{-1}) of the model fungicide pyrimethanil at 15, 20 and 25 °C revealed, that pyrimethanil caused concentration-dependent effects at all test temperatures. Interestingly, the ecotoxicity of pyrimethanil was higher at lower (suboptimal) temperature for embryo hatching and F_1 reproduction, but its ecotoxicity for juvenile growth and F_0 reproduction increased with increasing temperature.

The life-stage specific temperature-dependent ecotoxicity of pyrimethanil and the high fungicide susceptibility of the invasive snail clearly demonstrate the complexity of pesticide–temperature interactions and the challenge to draw conclusions for the risk of pesticides under the impact of global climate change.

1. Introduction

Increased temperature and dryness during summer months as predicted under global climate change (GCC) may induce land use changes (Bloomfield et al., 2006). An intensified use of agrochemicals is expected and additionally, predicted extreme weather events and thereby increased run-off may lead to an enhanced entry of pesticides into surface waters (Bloomfield et al., 2006; Schroll et al., 2006; Müller et al., 2010).

Fungicides represent a major class of pesticides used in agriculture (Berenzen et al., 2005). One of these fungicides is pyrimethanil which is currently used in apple orchards (application rate: 600 g ha^{-1}) and vineyards (application rate: 1 kg ha^{-1}) to combat mildew and gray mold. The current predicted environmental concentration of pyrimethanil in surface waters accounts to approximately 90 μg L^{-1} in apple orchards and 27 μg L^{-1} for vine cultures (EFSA, 2006). Further, the fungicide has frequently been detected in European surface waters with values up to 6.8–70 μg pyrimethanil L^{-1} or 272 μg kg^{-1} within sediments (Schlichtig et al., 2001; Verdisson et al., 2001; Kreuger et al., 2010; Schäfer et al., 2011).

The response of aquatic communities to the combined impact of agrochemicals and GCC will strongly depend on the reaction of aquatic key species (Heugens et al., 2001; Vinebrooke et al., 2004; Thuiller, 2007; Ferreira et al., 2010; Vandenbrouck et al., 2011). Knowledge on biological reactions and adaptation potential of species and communities toward stressor combinations is however insufficient (Kwok and Leung, 2005; Lannig et al., 2006; Oetken et al., 2009). The few available studies demonstrated that the toxicity of xenobiotics most often increases under the impact of higher temperature (Cairns et al., 1975; Gagné et al., 2007). A study conducted by Schäfer et al. (2007) showed that macroinvertebrate communities can be affected at low pesticide levels due to a combined synergistic impact of multiple natural stressors and pollution in the field. Further cumulative additive effects and non-additive interactions of natural antagonists and pollutants can result in remarkable impacts on ecologically relevant parameters (Coors and De Meester, 2005). Therefore to advance the knowledge about chronic impacts of chemicals on the aquatic environment, it is useful to expose key organisms for a longer time towards pollutants and monitor for their adaptive or sensitized responses (Bossuyt et al., 2005; Massarin et al., 2010; Staples et al., 2011;

* Corresponding author.
E-mail address: seeland@bio.uni-frankfurt.de (A. Seeland).

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Salice et al., 2010; Müller et al., 2012). Our recent research on pyrimethanil supports a positive toxicity–temperature relationship. For example, *Daphnia magna* (Straus, 1820) and *Chironomus riparius* (Meigen, 1804) being generally adapted to temperatures around 20 °C reacted more sensitive to low doses of the fungicide under increased temperature (Müller et al., 2012; Seeland et al., 2012). Conversely, other studies reported a negative correlation between temperature and toxicity. For example, pesticides like DDT and pyrethroids caused a higher ecotoxicity under lower temperatures (Gordon, 2005). To further increase the complexity, the reaction to multiple stressors is often species-specific (Gordon, 2005; Deschaseaux et al., 2010).

Besides, not only the pesticide or species under investigation is of decisive importance, even the investigated developmental stage may influence the outcome of ecotoxicological temperature experiments. Sawasdee and Köhler (2009, 2010) showed that it is of utmost importance to understand the sublethal effects of toxicants on various developmental stages. This finding contrasts the majority of multiple stressor research on fish and aquatic invertebrates, where the toxicity of pesticides is mainly examined in mature aquatic stages considering mortality (Hamilton, 1995; Clearwater et al., 2002; Grosell et al., 2006; Osman et al., 2007; Vieira et al., 2009; Yadav and Trivedi, 2009). To predict the impact of temperature and xenobiotics on the development of highly sensitive growth stages, it might be useful to examine eggs (Luckenbach et al., 2001; Osterauer et al., 2009). Particularly the often pellucid eggs of snails are promising to monitor stress effects on the development of embryos (Schirling et al., 2006; Sawasdee and Köhler, 2009). Gastropods are moreover highly relevant in freshwater ecosystems and fulfill all requirements of good bioindicators (Melo et al., 2000; Oehlmann et al., 2007; Das and Khangarot, 2011).

One omnipresent, dominant and invasive mollusc is the freshwater snail *Physella acuta* (Draparnaud, 1805) (Basommatophora, Physidae) living in streams, lakes and ponds (Bacchetta et al., 2001). *P. acuta* has a holarctic origin but meanwhile it is distributed worldwide, except of the polar regions. The success of the snail in conquering new habitats is based on its high reproduction rates, high passive dispersal capacities and an advanced tolerance against disturbed environments, pollution and high temperature (Brackenbury and Appleton, 1993; Albrecht et al., 2009). *P. acuta* is hermaphroditic and can reproduce uni- and biparentally (Sánchez-Argüello et al., 2009).

With regard to the uncomplicated monitoring of its life-stages, *P. acuta* is a very useful test organism to test the hypothesis if single and potential interactive effects of pyrimethanil and temperature differ between life stages. The hypothesis was tested by means of an embryo toxicity test, a juvenile growth test, a half- and full-life-cycle test (F_0 and F_1), which were conducted at a broad concentration range and three constant test temperatures.

2. Material and methods

2.1. Material

Physella acuta originated from our in-house culture (Institute for Ecology, Evolution and Diversity, Goethe University Frankfurt am Main). The snails were cultured in ISO medium according to the OECD guideline 202 (OECD, 2004) at a light:dark cycle of 16:8 h and 20 ± 1 °C. The water was renewed once a week and snails were fed two times weekly with Tetra Min® *ad libitum*.

The test substance pyrimethanil (Cas-No: 53112-28-0, PESTANAL®, analytical standard (99.9%)) was obtained from Sigma–Aldrich (Steinheim, Germany). The chemical behavior of pyrimethanil at three test temperatures was exemplarily measured in the concentration range of 0.15–2.5 mg L^{-1} during six days in accordance to the HPLC protocol for water samples published by Müller et al. (2012). The nominal pyrimethanil concentrations used for ecotoxicological bioassays ranged from 0.06 to 0.5 or 1.0 mg L^{-1} plus control and were received from effect concentrations determined in preliminary tests (for more information see Supplementary material).

2.2. Ecotoxicological bioassays

2.2.1. Embryo toxicity test at three temperatures

For the embryo test, eggs were isolated from 24 h-old egg masses by removing its surrounding gelatinous mass and separated into cavities of a 24-well plate. Each cavity was filled with 2 mL of either pure or pyrimethanil-spiked ISO medium. For each treatment, twelve replicates with one egg each were exposed to 15, 20 and 25 °C in climate chambers (MKKL 1200, Flohrs Instruments GmbH, Netherlands). Once a week the water was renewed. The experiment lasted for two weeks in minimum and for four weeks in maximum, depending on the temperature-dependent full hatching success of controls. The development of the embryos was daily documented with an inverse microscope (Axiovert 40c, Zeiss, Oberkochen) by means of the following endpoints: embryonic development stage (Fig. 1), day of hatching, hatching success, mortality, and anomalies. Hatching defined as the time point when an embryo had left the egg integument, while morphologic alterations compared to the control group (malformation, cessation of development, deformation/loss of the

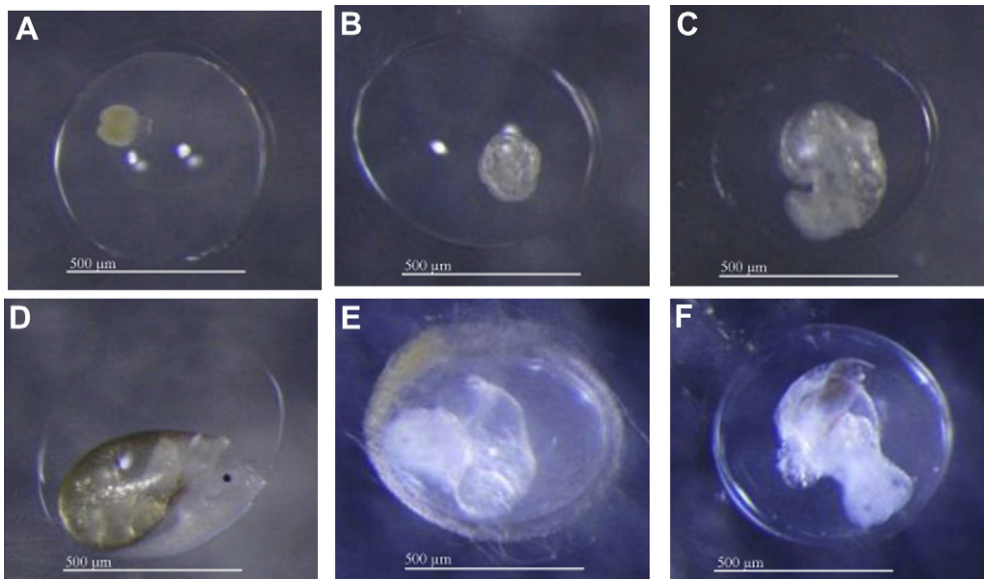

Fig. 1. Four embryonic developmental stages and deformation after exposure to pyrimethanil of *Physa acuta*. A = morula/gastrula (1st day), B = trochophora (4th day), C = veliger (6th day), D = hippo (8th day), E/F = edema and deformation of shell (0.25 mg L^{-1}).

shell) were summarized as anomalies. In addition the heartbeat rate (beats per minute, bpm) was determined after the heart had developed.

2.2.2. Juvenile growth test at three temperatures

For the assessment of the juvenile growth test freshly hatched juveniles were used. The juveniles were placed into 12-well-plates (equates twelve replicates), where each cavity contained 5 mL of the control or respective pyrimethanil solution. At the beginning of the six weeks experiment the shell length of the juveniles was measured (0.808 ± 0.004 mm) using a microscope and an image analysis system (Diskus version 4.50, Hilgers, Königswinter, Germany) and afterwards they were transferred to climatic chambers with temperatures of 15, 20 and 25 °C. Once a week, the shell length of juveniles was measured and documented. Likewise once a week, the water was renewed and snails were fed with Tetra Min®. Incipient with a size of approximately 1.5 mm (after three weeks of the beginning of the test), the snails were all transferred to 100 mL glass beakers which contained 50 mL of the respective control/pyrimethanil solutions and were covered with small glass lids. This was necessary to avoid that larger snails drop out.

2.2.3. Half- and full-life cycle tests at three temperatures

For the half-life cycle test fertile adult snails (F_0 generation) were exposed to 15, 20 or 25 °C. The snails were acclimated to the respective test temperatures for 14 d and exposed in five replicates with three snails per replicate for 28 d. Thereby, 250 mL glass beakers closed with a lid were used as test vessels, while the test medium was aerated via Pasteur pipettes. Once a week, the renewal of medium was followed by a new application of pyrimethanil. Twice a week, the snails were fed with 5 mg Tetra Min® per snail and day and in parallel, egg masses, detritus and dead snails were removed from the vessels. For further experiments the egg masses were transferred to 1 L glass beakers filled with the respective control/pyrimethanil solutions. The 1 L containers were again incubated at 15, 20 or 25 °C.

For the full-life-cycle-test, the resultant embryos (F_1 generation) were transferred to 10 L aquaria filled with the respective control/pyrimethanil solutions and exposed at 15, 20 and 25 °C. The medium was renewed and again spiked with pyrimethanil once a week and the snails were fed with Tetra Min® *ad libitum*. Once the F_1 snails were sexually mature again, the second full-life cycle test (F_1) was implemented as described above. Thereby, the egg masses produced by F_1 individuals were retained in 24-well-plates for monitoring the fertility.

2.3. Data analysis

Data were analyzed using the software programs Microsoft® Excel® and GraphPad Prism® (version 5.03) and Statistica® (version 7.1). If not stated differently, data is reported as mean [± standard deviation]. The 10% and 50% effect concentrations (EC_{10}, EC_{50}) for the toxicity tests were derived using a non-linear regression curve fit model. To calculate the no-observed-effect-concentration (NOEC) and the lowest-observed-effect-concentration (LOEC), all data sets were first checked for normal distribution with the D'Agostino- and Pearson-test ($n \geq 8$) or the Kolmogorov–Smirnov test ($n = 5–7$). In addition, percentage data were arcsine transformed and the homogeneity of variances was tested with Cochran's or Levene's test ($p < 0.01$). The ecotoxicity of pyrimethanil under different temperatures was analyzed using a two-factorial ANOVA followed by Tukey post-test ($p \leq 0.05$).

3. Results

3.1. Combined effects of pyrimethanil and temperature on embryonic development

The hatching time of the controls decreased (Fig. 2A) and the heartbeat increased significantly with increasing temperature. While the average heart rate was 67 bpm in the 15 °C control, it increased to 90 bpm at 20 °C and to 112 bpm at 25 °C. An additional exposure to pyrimethanil led to a delayed hatching and a faster heartbeat of the embryos (data not shown). But not only the individual stressors temperature and pyrimethanil, also their combination influenced the hatching time and the embryonic heartbeat significantly (Table 1). Similarly, temperature, pyrimethanil as well as their interaction highly influenced the degree of embryonic deformation and mortality (Fig. 1, Table 1). No mortality or embryonic deformations could be detected in the controls and in

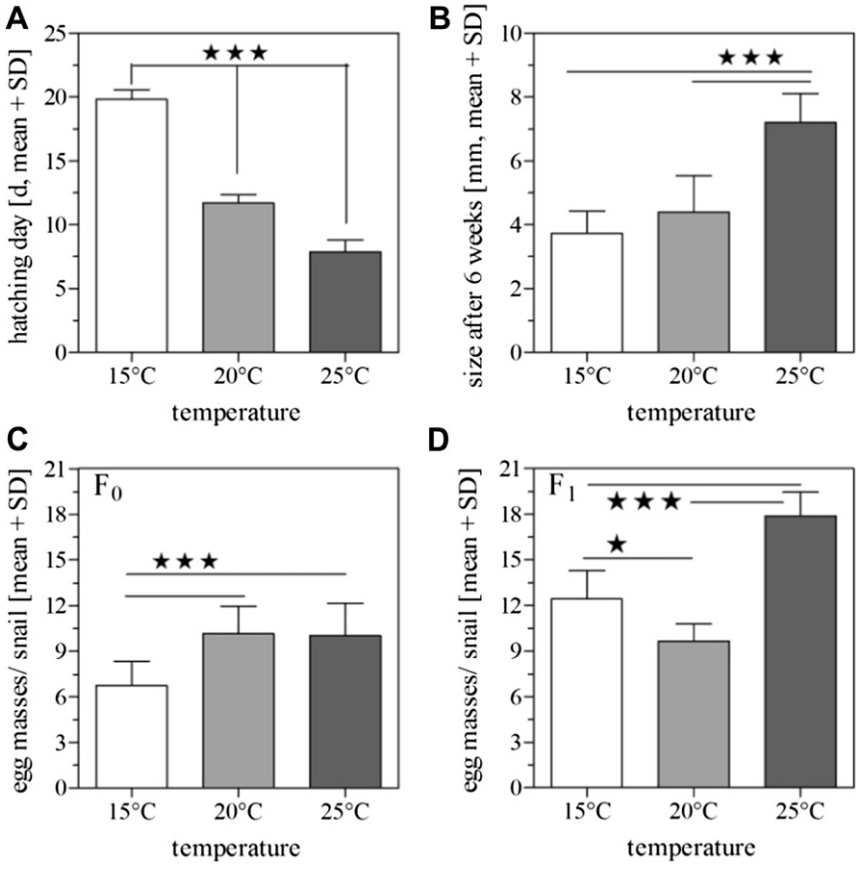

Fig. 2. A–D. Thermal response of developmental stages of *Physella acuta*. A: embryo development expressed as hatching day [d, mean + SD]; B: juvenile growth expressed as shell height [mm, mean + SD]; C: egg masses per snail produced by the F_0 generation [mean + SD]; D: egg masses per snail produced by the F_1 generation [mean + SD]. Significant differences: ★ = $p < 0.05$; ★★★ = $p < 0.001$.

Table 1
Individual and interactive effects of temperature (*T*) and pyrimethanil (Pyr) on the performance of embryos, juveniles, and adults of *Physella acuta* tested with a two-way ANOVA. The degrees of freedom, *F*- and *p*-values are shown. Significant differences: ★ = $p < 0.05$, ★★★ = $p < 0.001$.

Dependent variable	Factor	df	*F*-value	*p*-value	
Embryo toxicity test	*T*	2	45.531	<0.001	★★★
• Hatching	Pyr	5	131.76	<0.001	★★★
	T × Pyr	10	12.650	<0.001	★★★
• Deformation and mortality	*T*	4	2.5964	0.036	★
	Pyr	10	38.706	<0.001	★★★
	T × Pyr	20	2.6542	<0.001	★★★
• Heartbeat	*T*	4	7.0793	<0.01	★★
	Pyr	10	5.6894	<0.001	★★★
	T × Pyr	20	9.5717	<0.001	★★★
Juvenile growth test	*T*	2	48.050	<0.001	★★★
• Final size	Pyr	4	67.778	<0.001	★★★
	T × Pyr	8	12.611	<0.001	★★★
Half life cycle test	*T*	2	28.021	<0.001	★★★
• Egg masses per F_0 snail	Pyr	5	50.618	<0.001	★★★
	T × Pyr	10	2.2840	0.022	★
Full life cycle test	*T*	2	123.14	<0.001	★★★
• Egg masses per F_1 snail	Pyr	5	208.53	<0.001	★★★
	T × Pyr	10	20.065	<0.001	★★★

the lowest pyrimethanil treatments (60 μg L^{-1}) at all test temperatures (Fig. 3). But the higher the pyrimethanil concentration was, the higher the number of deformed or deceased embryos was regardless of the test temperature (Fig. 3). The number of embryos showing abnormalities (maximum = 53.8% at 0.25 mg L^{-1} and 15 °C) decreased at higher pyrimethanil concentrations while the mortality simultaneously increased. In the highest pyrimethanil test concentration (1.0 mg L^{-1}), the embryo mortality reached 91.7% at 15 °C, 83.3% at 20 °C and 100% at 25 °C indicating a trend for an increased ecotoxicity of pyrimethanil at higher temperatures. In contrast, the $EC_{10/50}$-values point to a decreasing toxicity of the fungicide with increasing temperature, although the confidence limits overlap strongly (Table 2).

3.2. Combined effects of pyrimethanil and temperature on juvenile growth

After six weeks, the final size of the juveniles was highly influenced by the single and combined impact of temperature and the fungicide (Table 1). The juvenile snails reached a size of 3.76 ± 0.69 mm at 15 °C if they were exposed to ISO medium only. At 20 °C the control snails grew to a size of 4.40 ± 1.13 mm, while the significantly strongest juvenile growth was observed in the controls at 25 °C (7.20 ± 0.91 mm) (Fig. 2B). Compared to the respective control treatments, the size of the juveniles significantly increased after exposure to pyrimethanil at 0.06–0.12 mg L^{-1} at 20 °C (47.2 and 43.8%), whereas no significant effects were observed in the range of 0.06–0.25 mg L^{-1} at 15 °C, and adverse effects on juvenile growth appeared after exposure to 0.06 and 0.25 mg L^{-1} at 25 °C (reduction by 35.2 and 34.8%) (Fig. 4). At 0.5 mg L^{-1}, the growth was significantly reduced compared to the control treatments at 15 °C (0.85 ± 0.22 mm, reduction by 83.4%) and 20 °C (1.35 ± 0.32 mm, reduction by 69.4%) and led to 100% mortality at 25 °C. This pattern clearly shows that the ecotoxicity of pyrimethanil on juvenile growth increased with increasing temperature, although the results of the EC_x calculations point to the opposite direction (Table 2).

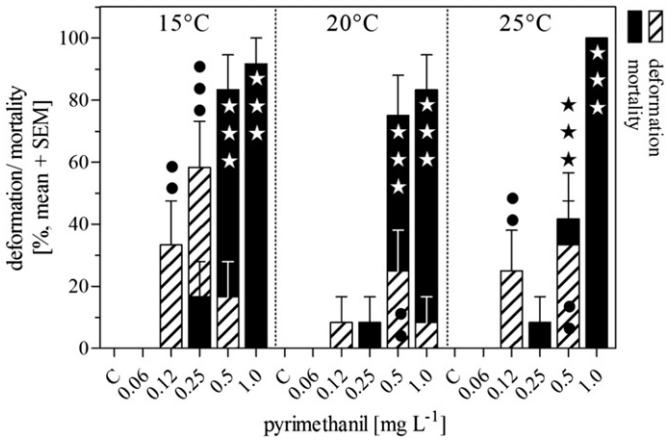

Fig. 3. Embryo toxicity test with *Physella acuta*. Deformation [%, mean + SEM] and mortality [%, mean + SEM] of embryonic snails after exposure to 0.06–1.0 mg L^{-1} of pyrimethanil and a control treatment at three temperatures (15 °C, 20 °C, 25 °C). Significant differences to control ($n = 12$): ●● = $p < 0.01$, ●●● = $p < 0.001$ for deformation; ★★ = $p < 0.01$; ★★★ = $p < 0.001$ for mortality. C = control.

3.3. Combined effects of pyrimethanil and temperature on F_0 survival and reproduction

The mortality of controls and pyrimethanil treatments in the F_0 snails tended to increase with increasing temperature, while the mortality within a certain temperature treatment (25 °C) achieved its maximum (59.6%) at the highest pyrimethanil concentration (Table 3). The reproduction of the F_0 survivors was likewise highly dependent on the temperature (Table 1, Fig. 2C). The amount of egg masses $snail^{-1}$ under control conditions significantly increased in response to rising temperature (6.74 ± 1.62 egg masses $snail^{-1}$ at 15 °C, 10.1 ± 1.79 egg masses $snail^{-1}$ at 20 °C, 10.1 ± 2.12 egg masses $snail^{-1}$ at 25 °C, Fig. 5A).

Pyrimethanil in the test media degraded slightly within six days, but similarly at all test temperatures (22.1 ± 8.9% at 15 °C, 14.8 ± 15.1% at 20 °C, 20.1 ± 13.0% at 25 °C). Hence the time weighted average pyrimethanil exposure for *P. acuta* was comparable at all temperatures (Table 4) during the regular interval of medium renewal in the test vessels. True for every temperature, egg mass production decreased in dependence on pyrimethanil concentration. In particular at concentrations higher than 0.25 mg L^{-1}, the egg mass production was strongly reduced (~factor 2–4). The ecotoxicological effect patterns were very similar between temperature regimes as indicated by the identical NOEC/LOEC-values calculated for every temperature treatment. Although the impact of the two single stressors seems to be dominant, temperature and the fungicide acted interactively (Table 1). Indeed the percentage of inhibition at the highest test concentration differed between temperature treatments with 72.7% at 15 °C, 80.9% at 20 °C and 80.6% at 25 °C. In opposite, the EC_{10}-values of the F_0 with 0.115 mg L^{-1} (15 °C), 0.200 mg L^{-1} (20 °C) and 0.292 mg L^{-1} (25 °C) implied a decreased pyrimethanil-toxicity at rising temperature.

3.4. Combined effects of pyrimethanil and temperature on F_1 survival and reproduction

First it should be noted that as a result of the poor reproduction of F_0 adults at 0.5 and 1.0 mg L^{-1}, a F_1 could not be established for these pyrimethanil treatments. Furthermore, large differences between the F_0 and F_1 life-cycle-tests became obvious. In contrast to the F_0, all adult snails of the F_1 survived the temperature and pyrimethanil exposure (Table 4). Moreover, if comparing the egg mass production between the two full-life-cycle tests, the F_1 adults in the controls and lower pyrimethanil treatments doubled at 15 °C (12.4 ± 1.86 egg masses $snail^{-1}$) and 25 °C (17.9 ± 1.56 egg masses

Table 2
NOEC, LOEC and $EC_{10/50}$ values [mg L^{-1}], and the percentage variance of the confidence interval derived from ecotoxicological test performed with different life stages of *Physella acuta* at three test temperatures (15 °C, 20 °C, 25 °C). Arrows point to an increased (↑) or decreased (↓) pyrimethanil toxicity or variance of the response in dependence on temperature, while the equal sign (=) indicates no temperature dependent ecotoxicity.

Test (endpoint)	NOEC [mg L^{-1}]	LOEC [mg L^{-1}]	EC_{10} ± CI [mg L^{-1}]	EC_{50} ± CI [mg L^{-1}]	CI [%]	
					EC_{10}	EC_{50}
Embryo toxicity test (mortality)						
	=	=	↓	↓	↑	↑↓
15 °C	0.25	0.5	0.218 ± [0.166–0.285]	0.355 ± [0.303–0.415]	54.6	31.5
20 °C	0.25	0.5	0.244 ± [0.169–0.353]	0.402 ± [0.339–0.475]	75.4	33.8
25 °C	0.25	0.5	0.346 ± [0.238–0.503]	0.529 ± [0.471–0.594]	76.6	23.3
Juvenile growth test (final size)						
	↑	↑	(↓)	(=)	(↑)	(↑)
15 °C	0.25	0.5	0.033 ± [0.016–0.067]	0.213 ± [0.160–0.283]	15.5	57.7
20 °C	<0.06	0.06	n.d.	n.d.	n.d.	n.d.
25 °C	<0.06	0.06	0.185 ± [0.022–0.123]	0.224 ± [0.162–0.309]	54.6	65.6
Half life cycle test (egg masses per F_0 snail)						
	=	=	↓	(=)	↑↓	(↓)
15 °C	0.25	0.5	0.115 ± [0.060–0.212]	0.403 ± [0.305–0.533]	132.2	56.6
20 °C	0.25	0.5	0.200 ± [0.133–0.302]	0.395 ± [0.327–0.477]	84.5	38.0
25 °C	0.25	0.5	0.292 ± [0.159–0.534]	n.d.	128.4	n.d.
Full life cycle test (egg masses per F_1 snail)						
	↑	↑	(=)	↓	↑	↑↓
15 °C	0.06	0.12	0.078 ± [0.065–0.092]	0.112 ± [0.109–0.116]	34.6	6.3
20 °C	0.12	0.25	0.102 ± [0.064–0.116]	0.204 ± [0.167–0.248]	51.0	39.7
25 °C	<0.06	0.06	0.090 ± [0.017–0.465]	2.371 ± [0.009–593.4]	497.8	24.7

snail^{-1}) (Figs. 2C, D, and 5). On the other hand, the reproductive power of the controls did not differ among the two life-cycle-tests at 20 °C (F_0: 10.1 ± 1.79 egg masses snail^{-1}, F_1: 9.66 ± 1.13 egg masses snail^{-1}).

The response of F_1 snails to pyrimethanil differed strongly from the F_0 at all test temperatures. At 20 °C, first significant effects were detected at 0.25 mg L^{-1}, which means at one concentration step lower than the LOEC assessed in F_0. The alteration of pyrimethanil ecotoxicity became more evident at 15 °C seeing that the snails died at 0.25 mg L^{-1} which was a pyrimethanil concentration without effects for the F_0. Moreover, at a concentration of 0.125 mg L^{-1}, the reproduction was severely inhibited if compared to the control (59.9%). Even at the optimal temperature of 25 °C (Fig. 2D), significant adverse pyrimethanil effects became apparent at low concentrations although the general high production level put the adverse effects at 25 °C into perspective. Nevertheless, the EC_{10}-values for the F_1 are in the same range for the three temperatures with 0.078 mg L^{-1} (15 °C), 0.102 mg L^{-1} (20 °C) and 0.090 mg L^{-1} (25 °C) which may be again reasoned by the overlapping confidence intervals.

The endpoint fertility of eggs generally mirrored the temperature and pyrimethanil effects observed for the endpoint egg mass snail^{-1} (cp. Table 4 and Fig. 5B). In brief, the highest fertility rate was detected in the control treatment at 15 °C, whereas the lowest fertility was observed at 0.25 mg L^{-1} and 20 °C (Table 4).

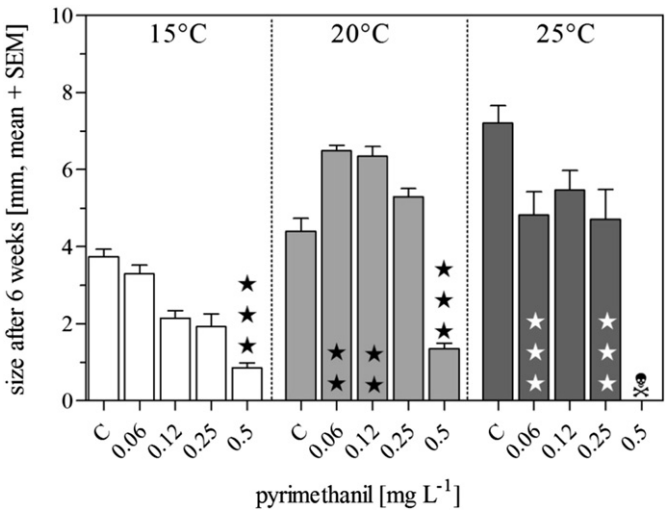

Fig. 4. Juvenile growth test with *Physella acuta*. Size of juvenile snails [mm, mean + SD] after six weeks of exposure towards 0.06–0.5 mg L^{-1} of pyrimethanil and a control treatment at three temperatures (15 °C, 20 °C, 25 °C). Significant differences to control ($n = 12$): ★★ = $p < 0.01$; ★★★ = $p < 0.001$. ☠ = 100% lethality.

4. Discussion

The thermal optimum of *Physella acuta* was reported to be between 20 and 30 °C (Thomas and McClintock, 1990; Brackenbury and Appleton, 1991; Albrecht et al., 2009). In accordance, the highest test temperature of 25 °C advanced the development of

Table 3
Mortality of adult organisms and the fertility of produced egg masses [%]. + = Poor reproduction (=too few egg masses available to asses a second generation); x = no data available.

	F_0			F_1		
	15 °C	20 °C	25 °C	15 °C	20 °C	25 °C
Mortality						
Control	0	6.6	26.4	0	0	0
0.06 mg L^{-1}	6.6	13.2	26.4	0	0	0
0.12 mg L^{-1}	6.6	6.6	39.6	0	0	0
0.25 mg L^{-1}	6.6	6.6	39.6	0	0	0
0.5 mg L^{-1}	6.6	19.8	46.4	x	x	x
1.0 mg L^{-1}	13.2	46.4	59.6	x	x	x
Fertility						
Control	Continuation to F_1			92.0	87.3	82.8
0.06 mg L^{-1}	(therefore no calculation of			81.5	85.8	86.5
0.12 mg L^{-1}	fertility)			76.9	78.4	76.9
0.25 mg L^{-1}				0	67.8	78.2
0.5 mg L^{-1}	+	+	+	x	x	x
1.0 mg L^{-1}	+	+	+	x	x	x

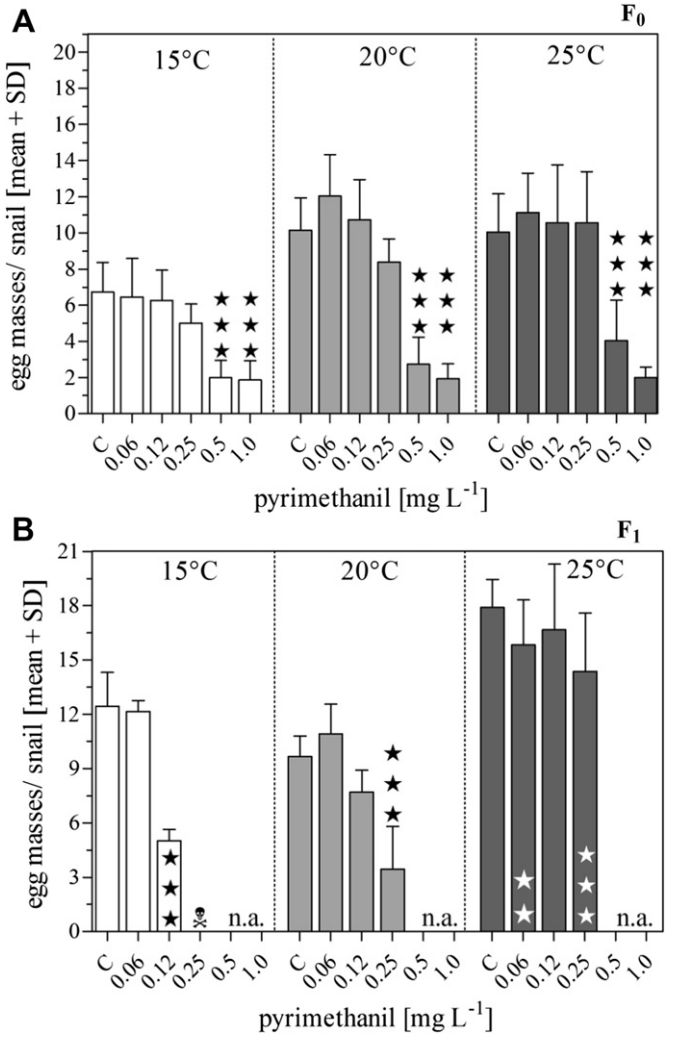

Fig. 5. Reproduction test with *Physella acuta*. Produced egg masses of each adult [mean + SD] after 28 days of exposure towards 0.06–1.0 mg L^{-1} (F_0) and 0.06–0.25 mg L^{-1} (F_1) of pyrimethanil and a control treatment at three temperatures (15 °C, 20 °C and 25 °C). A: reproduction of the F_0 generation, where only adults incurred fungicide exposure; B: reproduction of the F_1 generation, which parents were already exposed to the fungicide. Significant differences to control ($n = 5$): ★★ = $p < 0.01$; ★★★ = $p < 0.001$. C = control; ☠ = 100% lethality. n.a. = not available for the F_1. $n = 5$.

P. acuta with regard to the hatching of embryos, the juvenile growth and the reproduction of adult snails, while 20 °C was less beneficial for the species (Fig. 2). Nevertheless, the broad thermal tolerance of *P. acuta* reflects the good adaptation of the species to the thermal environment in small, shallow and even marginal water bodies with a low velocity which are all characterized by daily and seasonally strong temperature fluctuations (Thomas and McClintock, 1990). Moreover, the adaptation of *P. acuta* to high water temperatures might be of great advantage for its population dynamics under GCC.

Table 4
Time weighted average pyrimethanil concentration [mean ± SD] in the test medium after six days of exposure to 15, 20 or 25 °C. $n = 3$.

	0.15 mg L^{-1}	0.3 mg L^{-1}	0.6 mg L^{-1}	1.25 mg L^{-1}	2.5 mg L^{-1}
15 °C	0.14 ± 0.01	0.27 ± 0.04	0.56 ± 0.09	1.17 ± 0.16	2.63 ± 0.16
20 °C	0.15 ± 0.02	0.33 ± 0.05	0.63 ± 0.06	1.27 ± 0.14	2.48 ± 0.22
25 °C	0.15 ± 0.01	0.30 ± 0.03	0.64 ± 0.08	1.24 ± 0.16	2.52 ± 0.12

However, notwithstanding is the finding, that the F_0 adult snails in the control treatments revealed a considerably increased mortality at 25 °C, although no mortality could be observed in the F_1 adults at all temperatures (Table 4). The increased mortality of *P. acuta* adults in the F_0 may owe to the fact that the adult snails were taken from the breeding stock cultured at 20 °C for several generations and acclimated to the particular test temperatures for only 14 days. In contrast, the F_1 snails were exposed to the test temperatures from the very beginning of their embryonic life and might therefore be excellently thermally adapted. The differing thermal responses of two following generations denote the very slow-acting thermal acclimation of *P. acuta* – at least true for their adults – and should be considered in future temperature experiments.

In the context of GCC, aquatic species are not only affected by temperature alterations (Bicego et al., 2007; Schiedek et al., 2007). Although Bicego et al. (2007) mentioned that only few environmental factors have a comparable or even larger impact on animal energetics than temperature, the impact of pollutants as significant stressors for the aquatic fauna should be considered as well. Several studies showed that the ecotoxicity of xenobiotics frequently alter in dependence on the temperature (Brecken-Folse et al., 1994; Holmstrup et al., 2010; Müller et al., 2012). In accordance, temperature and pyrimethanil influenced the developmental stages of *P. acuta* interactively, but unexpected strongly and differently. The increased ecotoxicity of pyrimethanil on embryonic development and F_1 reproduction of *P. acuta* at 15 °C supports the hypothesis that the ecotoxicity of pollutants is most pronounced at species-specific suboptimal temperatures. Nevertheless, juvenile growth and F_0 survival and reproduction were most affected by the fungicide at 25 °C, although the increased F_0 reproductive inhibition owed obviously to the high control reproduction and the high F_0 mortality accounted most probably to an acclimation effect. However, if regarding only the embryo toxicity test, the juvenile growth test and the F_1-full-life-cycle test, the results clearly show, that a temperature–ecotoxicity relationship depends not only on the focused chemical or species as earlier reviewed by Cairns et al. (1975) and Heugens et al. (2001), but also differs among life-stages.

Pyrimethanil strongly inhibited the embryonic development, while the ecotoxicity increased with decreasing temperature (Fig. 3). True for all temperatures, first embryonic malformations emerged at 120 μg L^{-1} and were stepwise replaced by mortality at higher fungicide concentrations. Embryonic deformations are not a pyrimethanil-specific phenomenon since many environmental pollutants (*e.g.* endocrine disruptors, heavy metals, pesticides) can initiate malformations or other developmental changes in snail embryos (Hutchinson, 2002; Sawasdee and Köhler, 2009; Osterauer et al., 2011). The stronger pyrimethanil effect on the embryonic development at 15 °C (Fig. 3) might be well explained by a generally low metabolism and therefore very slow-running cell repair mechanisms at this suboptimal temperature. The assumption of low metabolic activity becomes strongly supported by the twofold prolonged developmental time observed at 15 °C (Fig. 2) and a slowed heartbeat (data not shown). Alternatively, the stronger pyrimethanil effects at 15 °C could be reasoned by the prolonged exposure of the embryos. However, the susceptibility of *P. acuta*-embryos towards pesticides will decrease under GCC conditions and thereby may support the invasive potential of the species. On the other hand in a hypothetic GCC scenario population size would be severely affected seeing that the juveniles responded more sensitive towards pyrimethanil than the embryos (Table 2).

This result contradicts the statement that early developmental stages of aquatic invertebrates are more sensitive than older ones (Gomot, 1998; Aguirre-Sierra et al., 2011). For instance all juveniles of *P. acuta* ceased at 500 μg L^{-1} at 25 °C, while the deformation and

mortality of embryos in this treatment counted altogether only ~45%. One possible reason for the reduced pyrimethanil susceptibility of embryos might be the egg integument. The integument protects the egg against harmful environmental influences, whilst the replacement of inorganic ions and water via the membrane can proceed (Beadle, 1969). Probably therefore and due to the fact that the whole body of juveniles is exposed towards the toxicant, effect concentrations calculated for juveniles by Oliveira-Filho et al. (2005) and in the present study were lower than those for eggs. Besides, the fungicide-temperature effect pattern of juvenile snails is more complex than that of the embryos, in particular at lower pyrimethanil concentrations (Fig. 4). Due to temperature impairing metabolism, movement and food intake of the juvenile snails (Smit and Van Gestel, 1997; Donker et al., 1998), it might be that an enhancement in growth coupled with an increasing pyrimethanil uptake leads to a higher mortality. Additionally, this effect might be enforced by their higher surface area to volume ratio which leads to a greater potential for agitation of water and ions across their surface (Kefford et al., 2004).

As earlier mentioned it is recognized that early developmental stages of most organisms are generally more sensitive to pollutants than older stages (Kefford et al., 2004). This is true for a part of our experiments, namely if the results of the juvenile growth test are compared with the F_0 of the full-life-cycle test. The exposed adult snails do not react as sensitively to pyrimethanil as the juveniles with EC_{10}-values of 0.115, 0.200 and 0.292 mg L^{-1}. However, the adult snails are more sensitive to pyrimethanil under the 15 °C treatment which is similar to the results of the embryo and juvenile tests. It is conceivable that the snails did not ingest as much food like those snails being exposed to 20 °C and 25 °C because their metabolic rate is reduced due to the low temperature being confirmed by food remains in the vessels. A lower food intake is followed by a low energy budget. This energy is however needed to compensate the disturbance of the homeostasis due to insufficient temperatures. Thus pollutants might become reduced and energy reserves may corrode (Calow and Sibly, 1990).

Energy consuming processes like repair and detoxifying mechanisms may have demanded for energy that otherwise could be used for offspring production. The egg mass production increased at 20 °C and 25 °C compared to 15 °C and independent of the pyrimethanil concentration, the production of egg masses was also enhanced if compared to the control. The stimulating effects of temperature are likewise illustrated by the study of Van der Schalie and Berry (1973). Their experiments with *Physa gyrina* (Say, 1821) showed that the amount of egg masses increased between 10 °C and 30 °C, with an optimum at 24 °C. In relation to the egg masses the optimal temperature is between 20 and 25 °C for *P. acuta* as well. That the energy reserves increase cannot only be seen for snails but for daphnids as well. Bergman-Filho et al. (2011) showed that natural stressors, like increasing temperatures, can cause alterations in the energy budget, in particular on protein and sugar contents. At the end these changes may result in modifications in individual growth or reproduction, changing at the end the whole population structure.

Most probably, the low fungicide concentration cannot retort the positive influence of the increasing temperatures. It has been shown for aquatic invertebrates that low NaCl concentrations in connection with high temperatures caused highest developmental rates in juveniles (Hall and Burns, 2002). Indeed, if certain concentrations of pollutants are exceeded the detoxification processes and retention of homeostasis consumes for a higher energy budget (Coutellec and Lagadic, 2006) more than available and therefore less egg masses are produced the higher the temperature and pyrimethanil levels are. This effect is enhanced by low oxygen concentrations at higher temperatures in the water bodies. Moreover, the positive correlation between toxicity and increasing temperatures might be accompanied by an increased uptake of the chemicals and sensitivity due to an enhanced metabolism rate (Gagné et al., 2007; Oetken et al., 2009; Holmstrup et al., 2010) followed in this case by high mortality rates of the adults (59.6%) in the F_0 at 1.0 mg pyrimethanil L^{-1} and 25 °C.

Numerous studies report a decrease in the fertility of snails due to xenobiotics (*e.g.* Coutellec and Lagadic, 2006; Oliveira-Filho et al., 2009; Das and Khangarot, 2011). Indeed the egg masses produced by the F_0 were not fertilized at 0.5 and 1.0 mg L^{-1} at all test temperatures, but not any fertilized egg masses were observed in F_1 adults at one concentration step lower (0.25 mg L^{-1}) at 15 °C. The suboptimal low temperature may have induced stress reactions like the damage of subcellular structures or the disturbance of the homeostasis (Halliwell and Gutteridge, 1985). This may have resulted in severe and long-lasting cellular damages and consequently led to unfertile egg masses after chronic exposure to the combined thermal and chemical stressors. All these mentioned factors may lead to sensitive responses during neonate and juvenile growth or a decrease in fecundity due to an intoxication from adults to progeny (Salice et al., 2009) as observed for the F_1 adults (Fig. A2). Similarly, a decline in the population level over several generations has been shown for heavy metals (Ducrot et al., 2007; Salice et al., 2009) and toxic microcystins produced by cyanobacteria (Lance et al., 2011).

Apart from the life-stage, the duration of exposure and/or the thermal acclimation status of the adult snail had apparently a strong effect on the pyrimethanil sensitivity and the fungicide-temperature-effect pattern concerning the reproduction of *P. acuta*. The general sensitivity for F_0 reproduction was at least doubled after a pyrimethanil-exposure of F_1 over several weeks as indicated by the NOEC/LOEC-values which decreased with decreasing temperature (Table 2). Oliveira-Filho et al. (2009) demonstrated likewise an increasing sensitivity of the F_1 of *Biomphalaria tenagophila* (Preston, 1910) after being exposed towards endosulfan or ethanol within a two-generation study. Moreover, the temperature–pyrimethanil relationship was not very conspicuous when considering the F_0 reproduction, while the ecotoxicity of pyrimethanil on the F_1 reproductive capacity was stressed at 15 °C (Table 1, Fig. 5). The different reserve capacities of adults may give the explanation for the lower sensitivity of the F_0 reproduction at 15 °C in the half-life-cycle test. The F_0 individuals could most probably resort to larger energy reserves to produce egg masses than the chronically stressed F_1 individuals. Other observations by Zalizniak and Nugegoda (2006) with *Daphnia carinata* (King, 1852) depict that the sensitivity of the parent generation can be in turn higher than the sensitivity being observed for the second generation.

In overall comparison, *P. acuta* considered to be tolerant against a broad range of stressors responded surprisingly sensitive towards pyrimethanil (Table 1) at an environmentally relevant concentration corresponding to the PEC. The chronic EC_{10} for the water flea *Daphnia magna* being the most sensitive ecotoxicological model organism was 0.94 mg L^{-1} (Seeland et al., 2012) within the 21 d reproduction test according to OECD 211 (OECD, 2008). Although that is in contrast to Von der Ohe and Liess (2004) who found snails not to be less sensitive as daphnids or several insects this concentration caused severe effects of pyrimethanil in *P. acuta* (Figs. 3–5). Similarly, *P. acuta* reacted significantly more sensitive than the model insect *Chironomus riparius* towards fluoxetine in a two-species water sediment test (Sánchez-Argüello et al., 2009). As a further example, long-term experiments with diquat unveiled the higher sensitivity of snails compared to ecotoxicological standard organisms (Ducrot et al., 2010). These studies may strongly support the inclusion of gastropods as well as full life cycle test into the

ecotoxicological test battery used in the aquatic pesticide risk assessment to better protect the aquatic environment from agrochemical pollution as it is suggested by OECD (2010) and Sieratowicz et al. (2011).

In addition, the future risk of pesticides for the aquatic community cannot be assessed by the recently available tools used for the regular risk assessment of agrochemicals as the impact of increasing temperature is not considered. In conclusion, the life-stage-specific ecotoxicity–temperature-relationship and the surprisingly high fungicide susceptibility of an invading, as eurYoecious classified gastropod complicates the agrochemical risk assessment for the aquatic environment in particular under GCC.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2012.10.020.

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