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Aquatic ecotoxicity of the fungicide pyrimethanil: Effect profile under optimal and thermal stress conditions

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ABSTRACT

The aquatic ecotoxic profile of the fungicide pyrimethanil and its acute and chronic thermal dependence in two aquatic invertebrates *Chironomus riparius* and *Daphnia magna* were investigated. The ecotoxicity of pyrimethanil at optimal thermal conditions did not depend on the trophic level, but was species-specific. The acute pyrimethanil-toxicity on *C. riparius* increased with higher temperature. The chronic response of *Daphnia magna* to the NOEC of the fungicide was examined in a multigenerational experiment under three near-natural temperature regimes. A pyrimethanil-induced increase of total mortality was buffered by the strongly related increase of the general reproductive capacity, while population growth was stronger influenced by temperature than by the fungicide. At a LOEC, however, a second generation could not be established with *D. magna* at all thermal regimes. This clearly shows that thermal and multigenerational effects should be considered when appraising the ecotoxicity of pesticides and assessing their future risk for the environment.

1. Introduction

Fungicides are used worldwide to control the development of pathogenic fungi on vegetables or fruits and to avoid crop failures due to fungal diseases (Ribeiro et al., 2000). For instance the grey mould *Bortrytis cinerea* can cause economically important damages on grapes, apples and tomatoes if not treated with fungicides (Latorre et al., 2002; Anfossi et al., 2006). One of the most frequently used fungicides in European vineyards is pyrimethanil (Navarro et al., 2000; Moyano et al., 2004; Anfossi et al., 2006). The application rates of pyrimethanil during the growing season are in average 600 g ha^{-1} in apple orchards or 1 kg ha^{-1} in vineyards (EFSA, 2006). Pyrimethanil is protectively applied up to five times during the growing season on plants and may reach the ground and surface water due to rain or spray drift (Verdisson et al., 2001; Mosleh et al., 2005; Anfossi et al., 2006; EFSA, 2006). It is well known, that pesticides as an extensively used class of environmental chemicals may impact natural ecosystems (Reinecke and Reinecke, 2007). In water courses nearby the application area, fungicides can affect the growth of non-target-species and disturb chemical and microbiological processes (Fernandez et al., 2005). In case of the regularly applied pyrimethanil, however, only little is known about its ecotoxicity, and mainly on aquatic primary producers (Tomlin, 1997).

A permanent reduction of the biodiversity in aquatic ecosystems may result from the global forecasted temperature increase of about 4 °C until the end of the 21st century in connection with an enforced use and entry of pollutants into the environment (Sala et al., 2000; Thuiller, 2007). At the same time, a significantly higher agricultural application of pesticides, especially fungicides, is required and might become usual in warmer and more humid climate expected to occur under climate change conditions (Boxall et al., 2009), which generally enforce fungal diseases as well (Anderson et al., 2004). Moreover, it is expected that temperature variations and changes in precipitation patterns towards extreme weather events like heavy rain fall during summer months will very likely cause a higher entry of contaminants such as agrochemicals into the aquatic environment (Noyes et al., 2009; Rosenzweig et al., 2001), mainly by influencing movement, partitioning, and distribution of chemical pollutants (Noyes et al., 2009; Schiedek et al., 2007). Moreover, it can be argued that thermal stress modifies the chemistry of pollutants (Schiedek et al., 2007) resulting under certain circumstances in a significant alteration of the ecotoxicological responses of aquatic species to pesticides (Heugens et al., 2001; Holmstrup et al., 2010). There are, however, only few experimental data to support this assumption for fungicides. In case of the fungicide fenarimol, the reproductive toxicity for the marine amphipod *Monoporeia affinis* increases with rising

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temperature, while the thermal and chemical stressors act synergistically (Jacobson et al., 2008).

As shown by Kim et al. (2010) it is important to take effects of relevant environmental conditions on the chemical toxicity into account in order to assess the ecosystem impact of contaminants more realistically. Abiotic and biotic parameters can influence the toxicity on aquatic organisms by up to an order of magnitude and furthermore, sometimes in not a consistent way. We therefore suggest that the toxic potential of the regularly applied fungicide pyrimethanil for aquatic key species may also change with increasing temperatures. To understand i) how toxic pyrimethanil is for different key species/trophic levels and ii) if the toxicity of pyrimethanil will change in the near-future aquatic environment under climate change this paper is divided in two parts.

The first part aims to test the effects of pyrimethanil on a broad range of aquatic model organisms to identify the most sensitive link in the ecosystem using a classical ecotoxicology approach. In the second part we investigate if and how temperature modulates the toxic potential of pyrimethanil in short-term and during a long-term exposure under either current or future, near-natural temperature conditions using an ecological approach. The selected temperature regime for our future scenario is within the forecasted climate change scenarios for European lakes as described by Mooij et al. (2007) and Thackeray et al. (2010).

2. Material and methods

2.1. Standard ecotoxicological tests

To determine the effect profile and toxicity of pyrimethanil on different trophic levels and systematic groups, literature was analyzed and supplement bioassays with standard organisms were applied as given in Table 1. All organisms tested in present study originated from in-house cultures of the Goethe University Frankfurt am Main and pyrimethanil (Cas No: 53112-28-0), PESTANAL®, analytical standard (99.9% purity)) was purchased from Sigma–Aldrich (Steinheim, Germany). In short, chronic pyrimethanil effects on algal/plant growth and on the mortality and reproduction of two invertebrates were supplementary studied. It has to be noted, that *Lumbriculus variegatus* was exposed in a sediment-water-system spiked with pyrimethanil via water phase (OECD 225, 2007). The sediment consisted of quartz sand and kaolin but contrary to the guideline no peat was added.

In addition, the acute toxicity of pyrimethanil for the survival of *Chironomus riparius* was tested according to OECD 235 (2011) with minor deviations. Therefore, 24 first instar larvae of *C. riparius* were introduced to 24-multiwell plates (2 mL spiked reconstituted water cavity^{-1}, pH 7.9–8.4, conductivity 540 μS cm^{-1}). One cethyl alcohol pellet was added to each well to reduce the surface tension. The larvae were exposed to two control (with/without cetyl alcohol) and seven pyrimethanil treatments at a light:dark cycle of 16:8 h under dim light without feeding at 20 ± 1 °C. After 48 h the larvae were checked for their mobility.

2.2. Pyrimethanil exposure under different temperature regimes

Further acute and chronic bioassays were conducted under different temperature regimes with pyrimethanil-sensitive test organisms. The acute impact of temperature on the ecotoxicity of pyrimethanil was studied with first instar larvae of *C. riparius* which were exposed in two control groups (with/without cetyl alcohol) and seven concentrations ranging from 0.5 to 32 mg L^{-1} of the fungicide at four static temperatures (14 °C, 18 °C, 22 °C and 26 °C) for 48 h (for details see 2.1). Beyond this acute response test we investigated how population dynamics of *Daphnia magna* respond to a chronic exposure to sublethal concentrations of the fungicide under near-natural, rapidly changing environmental conditions occurring today and in future. In this 140-days-lasting multigenerational study, *D. magna* was exposed to pyrimethanil at three different temperature scenarios: (I) 'cold year, today' (CYT), (II) 'warm year, today' (WYT) and (III) 'warm year, 2080' (WYF). Every climate scenario simulated in environmental chambers was arranged with a control treatment, a treatment with the NOEC (0.5 mg L^{-1}) and the LOEC (1.0 mg L^{-1}) for pyrimethanil determined in the standard reproduction test before.

The simulated and 20-minutely logged (TL20 loggers, AMZ Großhandels KG, Mainhausen, Germany) temperature regimes are given for each climate scenario in Fig. 1. The simulated present-day temperature regimes (Δ 10.8 °C) and the irradiation periods represent natural conditions typical for mid-April to mid-October (day 105–287 of a year). Dynamic test temperatures for both present-day scenarios 'cold year, today' and 'warm year, today' were based on field-measured water temperatures between 1990 and 2005 from lake Mainflingen and the river Main near Nied and Seligenstadt (Germany) (open source data, HLUG, 2010, Fig. 1). To simulate prospective water temperatures in ~2080 we used the calculated heating rates of 0.04–0.05 C yr^{-1} as observed in shallow lakes in Europe over the last forty years (Mooij et al., 2008; Thackeray et al., 2010). As a result, a mean temperature increase about 2.5 °C is expected for shallow water bodies in northern Europe till 2080 and is added on top of the temperatures for a 'warm year, today''. Changes in temperature for each scenario were manually adjusted every day by 0.2 °C. The starting temperature was 11.0° for CYT, 14 °C for WYT and 16.5 °C for the WYF. The mean temperature for each generation during 21 d is given in Table 2.

The constitutive experimental design followed the OECD guideline 211 for the reproduction test with *D. magna* (OECD, 2008). Always the third brood of the parental generation was used to establish a subsequent generation. Therefore all juvenile daphnids of the third brood from a given treatment were pooled and ten of them established the next generation. Every single generation has remained for 21 d in the test, so that the number of neonates per generation and for each temperature scenario could be compared. Two times weekly the test medium was renewed and the daphnids were fed with an algae suspension of *Desmodesmus subspicatus* (0.2 mg C daphnid^{-1} d^{-1}). As shown by Müller et al. (2012) there is only a slight decrease in the amount of pyrimethanil over 19 days which is furthermore not temperature-dependent. Therefore it was decided not to analyze the concentration of pyrimethanil within the water phase of the test vessels as the medium is renewed every three days.

2.3. Data analyses

If not stated differently, data are reported as mean [± standard deviation] and calculated with the software programs Excel® (Microsoft) and Prism® (version 5.03, GraphPad). The 10% and 50% effect concentrations (EC_{10}, EC_{50}) for the acute and chronic toxicity tests were derived using a non-linear regression curve fit model ($x = \log(x)$). To calculate the no-observed-effect-concentration (NOEC) and the lowest observed effect-concentration (LOEC), all data sets were checked for normal distribution with the D'Agostino and Pearson test ($n \geq 8$) or the Kolmogorov–Smirnov test ($n = 5–7$). Homogeneity of variances was tested with Bartlett's test at $p \leq 0.05$. The ecotoxicity of pyrimethanil was analyzed using a one-way ANOVA (F, $p \leq 0.05$) and subsequently a Dunnett's post hoc test. If the assumption for an ANOVA was not given, the Kruskall–Wallis-test followed by Dunn's post hoc test was used ($p \leq 0.05$). For the comparison of two means (e.g. acute immobilisation test with *D. magna*), the unpaired *t*-test for data sets with normal distribution or the Mann–Whitney-test was applied. In addition, the produced neonates of the pyrimethanil treatment and the control of a dedicated

Table 1
Conducted bioassays to assess the aquatic ecotoxicity of pyrimethanil. The organisms, endpoints, OECD guideline number, test duration and used concentrations are given. *modified as described in Material and Methods.

Test organism	Test system • Endpoint	OECD guideline	Test duration	Concentration range [mg L^{-1}]
Desmodesmus subspicatus	Growth inhibition test • Number of cells	201 (OECD, 2002)	72 h	0.6–50
Lemna minor	Growth inhibition test • Number of fronds	221 (OECD, 2006)	7 d	1.25–80
Lumbriculus variegatus	Sediment-water toxicity test • Number of worms	225 (OECD, 2007)	28 d	0.25–8.0
Chironomus riparius	Acute immobilisation test • Number of immobile individuals	235* (OECD, 2011)	48 h	0.5–32
Daphnia magna	Acute immobilisation test • Number of immobile individuals	202 (OECD, 2004)	48 h	0.25–16
D. magna	Reproduction test • Number of offspring	211 (OECD, 2008)	21 d	0.03–2.0

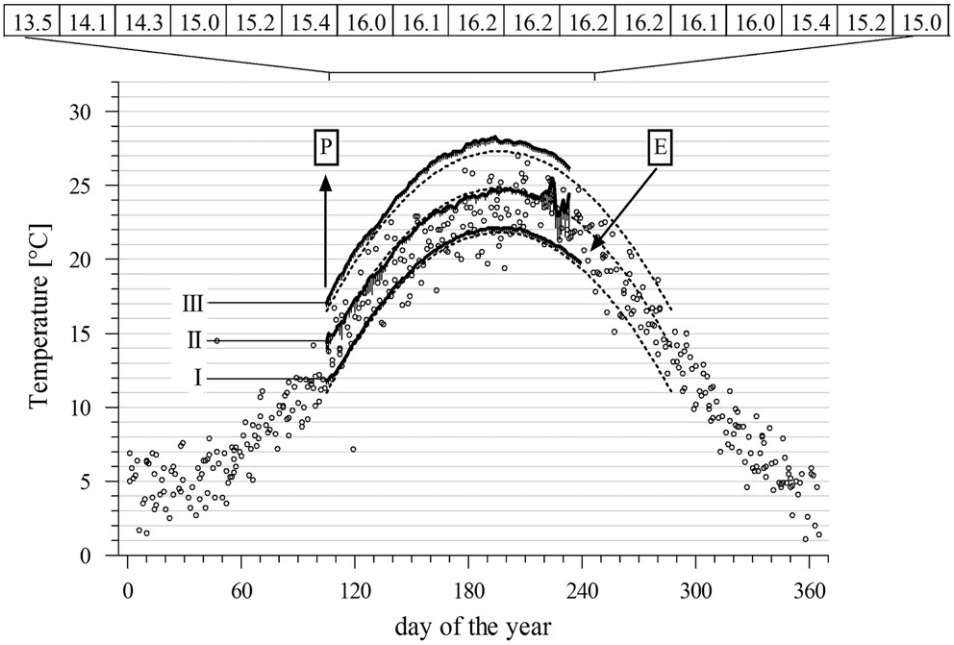

Fig. 1. Water temperature (open circles) measured at two sites of a large, slow flowing river (Main, Germany) and in a non-stratified pond with maximal 6 m depth (nature protection area Mainflingen, Germany) during 1990–2005 (data from HLUG, 2010). Fitted temperature curves (dashed lines, $y = -0.0013x^2 + 0.51x - b$) for a cold year (I, b = 22.72) and a warm year (II, b = 25.22) in 1990–2005 as well as expected for a warm year in ~2080 (III, b = 28.10) were used to simulate the period middle of April until end of August during the multigenerational study. Actually daily mean temperatures logged in environmentally controlled cabinets (bold lines, mean ± SD) from exposure of parental generation (P) of *Daphnia magna* until the end of experiment (E) are shown for comparison. Duration of irradiation [hours d^{-1}] was weekly adjusted (upper oblong).

generation were compared using a two-way ANOVA followed by Bonferroni post-test ($p \leq 0.05$).

To test for a linear relationship of pyrimethanil exposure on survival (acute test) or reproduction (multigenerational study) and (mean) temperature, linear regression analyses including Runs-test were accomplished. The same analysis was performed to test for a coherence of reproductive patterns and mortality during multiple generations. With regard to the ecological significance of the observed results, the intrinsic rate of population increase *r* was moreover calculated for each treatment and generation of the multigenerational study. The population growth rate *pgr* ($\triangleq r$) was iteratively calculated for each generation and treatment (Hammers-Wirtz and Ratte,

Table 2
Ecotoxicity of pyrimethanil [mg L^{-1} ± 95%-confidence interval] on aquatic model organisms and the respective endpoint. Effect concentrations are derived from standardized test systems and represent own data if not specified differently. NOEC = no observed effect concentrations, LOEC = lowest observed effect concentrations, LC_{10}/EC_{10} = concentration causing 10% mortality/effect, LC_{50}/EC_{50} = concentration causing 50% mortality/effect, CI = confidence interval.

Species	Endpoint	NOEC	LOEC	LC_{10}/EC_{10} [CI]	LC_{50}/EC_{50} [CI]
Primary producers					
Desmodemus subspicatus (chronic, 72 h)	growth	5	10	6.83 [5.60–8.32]	13.7 [12.2–15.3]
Scenedemus acutus (chronic, 48 h)	growth				23.1[a] [16.5–27.5]
S. obliquus (chronic, 96 h)	growth		0.2[b]		
D. quadricauda (chronic, 96 h)	growth	0.6[b]	0.8[b]		
Raphidocelis subcapitata (acute, 72 h)	growth				1.2[c]
Lemna minor (chronic, 7 d)	growth	2.5	5	3.07 [2.31–5.93]	23.4 [20.7–26.4]
L. minor (chronic, 6 d)	growth			3.60 [3.12–4.25][a]	46.1 [40.2–52.0][a]
L. gibba (chronic, 7 d)	biomass				7.8[c]
Primary consumers					
Lumbriculus variegatus (chronic, 28 d)	reproduction	4	8	1.52 [0.68–3.40]	12.7 [6.42–25.0]
Chironomus riparius (acute, 48 h)	immobility	0.5	1	0.91 [0.09–9.16]	2.92 [1.11–7.69]
C. riparius (chronic, 28 d)	emergence	4[d]			
C. riparius (chronic, 28 d)	mortality	4[e]	8[e]	5.38 [4.17–6.93][e]	9.27 [8.20–10.5][e]
Daphnia magna (acute, 48 h)	immobility	2	4	1.02 [0.48–2.19]	3.61 [2.62–4.97]
Secondary consumer					
D. magna (acute, 48 h)	immobility				2.9[d]
D. magna (chronic, 21 d)	reproduction	0.5	1	0.95 [0.67–1.35]	1.18 [0.42–3.32]
D. magna (chronic, 21 d)	reproduction	0.94[d]			
Oncorhynchus mykiss (acute, 96 h)	mortality				10.6[c]
O. mykiss (chronic, 21 d)			1.6[c]		

[a] Verdisson et al. (2001).
[b] Dosnon-Olette et al. (2010).
[c] PPDB (2009).
[d] EFSA (2006).
[e] Müller et al. (2012).

Table 3
Mean temperature, mortality and offspring number of *Daphnia magna* during the multigenerational study. Mean temperature [°C] during 21 d, mortality [%] of ten mother daphnids and number of neonates per daphnid [mean ± SD] within 21 days are given for every generation (Gen) of control and pyrimethanil treatments (0.5 mg L^{-1}) in three temperature scenarios. Bold numbers indicate significant differences between the control and pyrimethanil group of a certain generation.

Endpoint	Gen	Cold year, today		Warm year, today		Warm year, future	
		Control	Pyrimethanil	Control	Pyrimethanil	Control	Pyrimethanil
Mean temperature [°C] during 21 d	F_0	13.8	13.8	16.2	16.2	18.7	18.7
	F_1	17.9	16.9	19.9	20.2	21.6	21.7
	F_2	20.1	202	22.1	22.3	23.9	23.9
	F_3	21.2	21.2	23.4	23.6	25.4	25.4
	F_4	21.7	21.7	24.3	24.4	26.2	26.3
	F_5	21.7	21.7	24.7	24.7	26.8	26.9
	F_6	21.2	21.2	27.7	24.7	27.2	27.2
	F_7			24.5	24.2	27.2	27.2
	F_8					26.9	26.8
	F_9					26.4	26.2
Mortality	F_0	0	0	0	0	0	0
	F_1	0	20	0	0	0	0
	F_2	20	20	10	30	10	20
	F_3	0	0	10	20	0	20
	F_4	20	10	0	10	0	20
	F_5	0	10	20	10	0	30
	F_6	0	10	10	20	20	0
	F_7			0	30	50	40
	F_8					0	20
	F_9					0	10
	Mean	5.7	10.0	6.25	15.0	8.0	16.0
Neonates per daphnid	F_0	15.9 [6.20]	15.4 [5.58]	33.6 [10.0]	30.7 [11.6]	79.5 [7.33]	63.2 [8.12]
	F_1	76.3 [20.3]	59.1 [20.9]	**107 [21.0]**	**69.9 [14.8]**	**100 [22.0]**	**75.2 [18.9]**
	F_2	101 [15.2]	81.4 [28.8]	**154 [16.5]**	**96.4 [26.5]**	**139 [25.9]**	**92.9 [24.7]**
	F_3	97.1 [17.3]	86.9 [17.2]	121 [27.2]	109 [21.7]	155 [19.8]	138 [17.9]
	F_4	117 [12.9]	99.4 [21.9]	109 [17.1]	100 [16.8]	151 [31.1]	127 [16.4]
	F_5	109 [16.9]	112 [10.1]	**136 [18.2]**	**104 [16.7]**	99.0 [17.1]	94.0 [20.4]
	F_6	**94.5 [15.7]**	**65.0 [26.0]**	**147 [28.7]**	**96.4 [19.6]**	111 [25.2]	113 [12.9]
	F_7			112 [14.9]	105 [11.8]	146 [29.5]	137 [6.11]
	F_8					**148 [14.3]**	**113 [20.2]**
	F_9					98.4 [13.2]	94.6 [18.0]
	Median	97.1	81.4	116.5	98.2	125.0	103.8

2000; Muyssen and Janssen, 2004; Coors and De Meester, 2008) and further analyzed by means of a non-linear regression using a polynomial equation of the third order.

3. Results

3.1. Aquatic effect profile of pyrimethanil

Table 2 gives an overview of the observed and calculated effect concentrations reported in the literature or derived from our experiments which are described in detail in the appendix. NOEC values range from 0.50 to 5.0 mg L^{-1}, LOEC values from 0.20 to 10.0 mg L^{-1}, EC_{10} values from 0.95 to 6.83 mg L^{-1}, and EC_{50} values from 1.18 to 46.1 mg L^{-1}. The toxicity range and the variation of ecotoxicological values within the groups primary producers and consumers are comparable. Hence the ecotoxicity of pyrimethanil is species-specific and not dependent on the trophic level.

3.2. Combined effects of temperature and pyrimethanil

To test if the ecotoxicity of pyrimethanil depends on temperature, the pyrimethanil-sensitive species *Chironomus riparius* and *Daphnia magna* were exposed towards pyrimethanil at either constant or dynamic temperature regimes. The calculated effect concentrations (EC_{10} and EC_{50}) for survival of the *C. riparius* larvae at 14 °C–26 °C reveal that the ecotoxicity increased with rising temperatures (EC_{10}: $R^2 = 0.99$; EC_{50}: $R^2 = 0.98$; Fig. 2). The negative relationship of effective concentrations and temperature is more pronounced at higher concentrations (slope of $EC_{10} = -0.32 \pm 0.02$, slope of $EC_{50} = -0.73 \pm 0.06$).

To investigate if a chronic exposure to a NOEC or LOEC of pyrimethanil affects *D. magna* during multiple generations under near-natural temperature conditions, a multigenerational study was conducted. The daphnids exposed to the LOEC (1 mg L^{-1}) did not produce a F_1-generation in any of the temperature scenarios due to low reproduction rates and a high mortality within 21 d at the beginning of the experiment. However, under NOEC (0.5 mg L^{-1}) exposure, the population growth continued for four months under present and forecasted temperature regimes. During the multigenerational study, six generations were produced in the 'cold year,

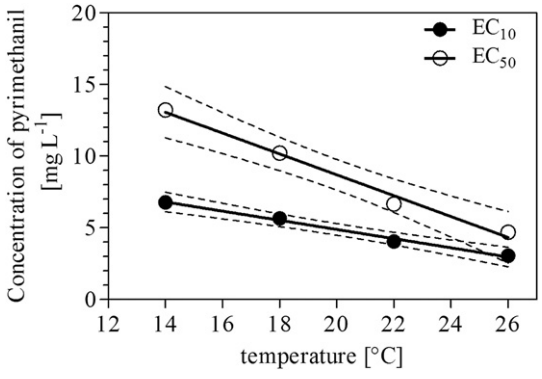

Fig. 2. Effective concentrations (EC_{10}, EC_{50}) of pyrimethanil for the acute immobility of first instar larvae of *Chironomus riparius* at four different temperatures. Lines represent linear regressions with 95% confidence intervals. Test duration = 48 h; 24 replicates per treatment.

today'. In the 'warm year, today' and 'warm year, 2080' daphnids produced 7 and 9 generations, respectively.

The number of neonates which were produced by control daphnids (Table 3) followed in general the temperature gradient ($R^2 = 0.63$, slope $= 7.62 \pm 1.23$; Fig. 3A). This was especially true for daphnid reproduction under the 'cold year, today' simulation. In contrast, the number of offspring of controls observed in the 'warm year, today' and 'warm year, 2080″ often deviated from experimental temperature profiles, in particular at temperatures above 21 °C. For the scenario 'warm year, today' the number of neonates decreased from generation F_2 to F_4 and after a maximum during F_6, again in F_7 (Table 3). Under 'warm year, 2080' conditions, reproduction was reduced in generations F_5. For generations F_7 and F_8 we observed a second reproduction maximum followed by a decline in generation F_9. More generally, reproduction of controls decreased clearly in the consecutive generations of both scenarios if a maximum amount of approximately 150 neonates per adult was reached. With the ongoing of the study, reproduction increased again and adult daphnids produced as many juveniles as in the first maximum. This bimodal pattern was most pronounced in the 'warm year, 2080' scenario.

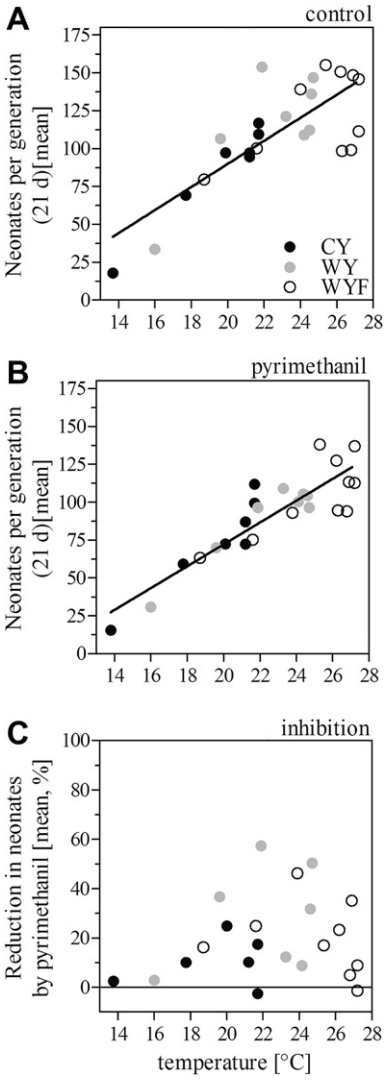

Fig. 3. Linear regression for the influence of temperature on the number of neonates (A and B) and ecotoxicity of pyrimethanil (C) during a 140-day-long multigenerational study with *Daphnia magna*. Each circle represents the number of neonates (A and B) due to increasing temperatures and accordingly the inhibition of neonates caused by pyrimethanil (C). Black dots = results for a 'cold year, today'; grey dots = 'warm year, today'; white dots = 'warm year, 2080'.

The NOEC of pyrimethanil inhibited the reproduction (in total average 19.9%) (Fig. 3C), which indicated a comparable level of effects of ca. 22% as observed at the NOEC in the standard test (Fig. A1–D). The most significant inhibitory effect of pyrimethanil on reproduction was lower under the 'cold year, today' simulation (maximum 31.2% in F_6) compared to the other scenarios. Under 'warm year' and 'warm year, 2080' simulations, the relative effect of pyrimethanil on reproduction increased significantly up to 37.4% (F_2) and 33.2% (F_2), respectively, compared to the control group. Although the reproduction under pyrimethanil exposure followed the temperature gradient similar to the controls ($R^2 = 0.79$, slope $= 7.22 \pm 0.79$), the percentage inhibition by pyrimethanil did not depend on the thermal history ($R^2 = 0.03$, $p = 0.44$, Fig. 3B, C). If the mean values of neonates over all generations are compared for the several temperature scenarios they are all in the same range with 16.2% for the 'cold year, today', 15.7% for the 'warm year, today' and 17.0% for the 'warm year, 2080' (Table 2).

Significant differences between the control and pyrimethanil treatments exposed to the 'warm year, today' and warm year, 2080' became mainly apparent, albeit only if the reproductive output in the control was short before or at peak reproduction with approximately 150 neonates per adult. Pyrimethanil-exposed daphnids had a lower maximum production per parent under 'warm year, today' (max. 109 juveniles) and 'warm year, 2080' conditions (max. 138 juveniles) compared to the control (max. 154 and 155 juveniles, respectively). If exposed to pyrimethanil, a bimodal reproductive response pattern as observed in the controls became exclusively obvious in the future 'warm year, 2080' scenario.

The combined effect of low fungicide exposure and thermal stress increased the average mortality as shown in Table 3. On the one hand mortality increased the higher the temperature was and on the other one the mortality was always approximately doubled if the daphnids were exposed towards pyrimethanil. The average mortality and the sum of neonates were positively correlated (controls: $R^2 = 0.77$; pyrimethanil treatments: $R^2 = 0.95$) and correspondingly, the impact of the fungicide on the intrinsic population growth rate was minor (Fig. 4A–C). The *pgr* increased with increasing temperatures. In the F_0 generation, the *pgr* of controls was 0.16 d^{-1} in the 'cold year, today', 0.21 d^{-1} in the 'warm year, today' and 0.30 d^{-1} in the 'warm year, 2080'. If temperatures achieved the highest temperature during the lapse of the experiment, the *pgr* reached 0.39 d^{-1} in the 'cold year, today' (F_4), 0.44 d^{-1} in the 'warm year, today' (F_5) and 0.50 d^{-1} in the 'warm year, 2080' (F_8).

4. Discussion

The ecotoxicity of pyrimethanil on aquatic model organisms does not depend on the trophic level, but is species-specific. Our study clearly shows that data from standard tests performed under optimal conditions underestimate the toxic potential of pyrimethanil under thermal stress conditions. Temperature is a strongly modifying factor for the acute ecotoxicity of pyrimethanil, particularly at higher concentrations. By the example of *Daphnia magna* it could be demonstrated that even an exposure to a NOEC of pyrimethanil significantly affects reproduction and to a lesser extent population growth rate (*pgr*), particularly under warm summer conditions.

Pyrimethanil causes adverse effects on aquatic primary producers. However, the interspecific variability of its ecotoxicity is high (Table 2; c.f. Verdisson et al., 2001; PPDB, 2009; Dosnon-Olette et al., 2010). Pyrimethanil effect concentrations vary within the

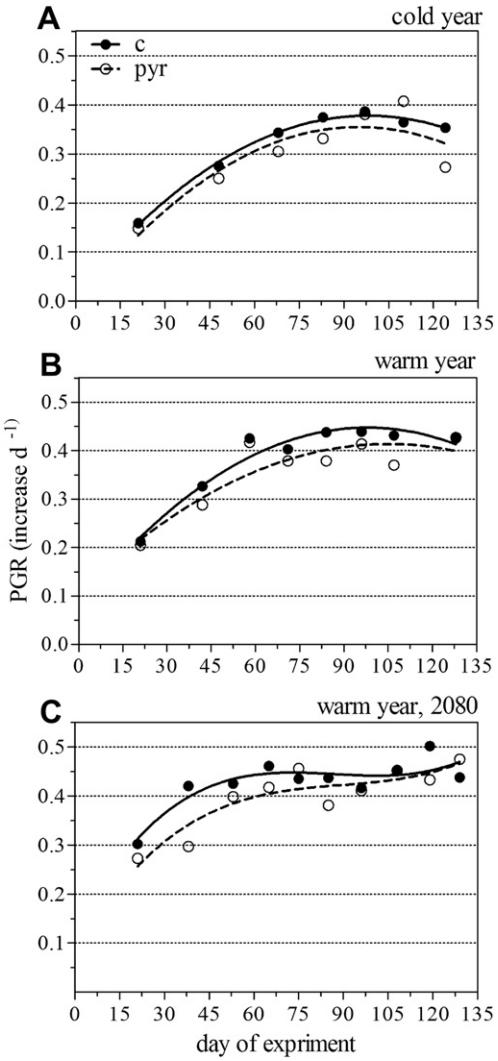

Fig. 4. Effects of pyrimethanil (0.5 mg L^{-1}) on the intrinsic population growth of *Daphnia magna* under dynamic changing temperature regimes [°C] during a 140-day-long multigenerational study. Each symbol (black dot = control, open dot = fungicide) represents the calculated increase of the growth rate for every generation (mean, $n = 10$). Temperature scenarios are A) 'cold year, today', B) 'warm year, today' and C) 'warm year, 2080'.

green algae by a factor ~20 if considering the EC_{50} values (Table 3). Belgers et al. (2009) propose that the risk assessment of fungicides should also cover macrophytes due to the difficult prediction of potential fungicidal effects on the environment. The ecotoxicological standard representatives for vascular aquatic plants, the Lemnaceae, respond however as sensitive towards pyrimethanil as the green algae, while available EC_{50} values for the species *L. minor* vary slightly about a factor of 2 (Table 3, Verdisson et al., 2001). The impairment of the photosynthetic system seems to be a general effect of pyrimethanil on aquatic primary producers as not only reported for Lemnaceae, but also for several other macrophytes and green algae (Appendix; Verdisson et al., 2001; Dosnon-Olette et al., 2009, 2010).

One may expect an increased ecotoxicity of pyrimethanil on primary consumers as Dosnon-Olette et al. (2009, 2010) provide evidence for pyrimethanil accumulation in algae and water plants. The ingestion of pyrimethanil-contaminated algae or detritus might be an additional, indirect pyrimethanil exposure route for detritivorous and herbivorous species. *Lumbriculus variegatus* and *Chironomus riparius* (larvae) are directly exposed to xenobiotics in the water and sediment due to their benthic mode of life (Pinder, 1986; Mosleh et al., 2005). The chronic exposure of *L. variegatus* and *C. riparius* to pyrimethanil has, however, no negative impact up to a concentration of 4 mg L^{-1} (Fig. A1–C, Table 3). Hence both detritivorous species are as susceptible as the primary producer *D. subspicatus* and the secondary consumer *Oncorhynchus mykiss*, for example (Table 3).

Nevertheless, the provided food during the bioassay with *L. variegatus* was not pre-contaminated with pyrimethanil and quickly ingested. Therefore an indirect exposure route of pyrimethanil can be excluded for *L. variegatus*. *D. magna* turned out to be a more sensitive standard organism towards pyrimethanil (Fig. A1–D; Table 3; EFSA, 2006). The susceptibility of *D. magna* toward fungicides might be in part based on the uptake of contaminated food and hence a result of the contemporary direct and indirect fungicidal exposure. During the chronic standard bioassay, *D. magna* was fed on fresh *D. subspicatus* algae every three to four days. Admittedly within four days, 7–10% of pyrimethanil disappeared from the medium in presence of the sister species *S. obliquus* and *D. quadricauda* (Dosnon-Olette et al., 2010). Therefore, the likely absorbed or bio-accumulated pyrimethanil in algal cells may have indirectly increased the ecotoxicity on *D. magna*. Supportively Taylor et al. (1998) demonstrated that Cd-contaminated algal cells depress the feeding rate of *D. magna* in a Cd-free medium due to the algal surface-bound fraction of Cd-ions. Furthermore, Bertram and Hart (1979) provide evidence that the reproduction of daphnids becomes affected due to Cd-contaminated food.

The impact of pyrimethanil on *D. magna* increases from acute to chronic exposure and finally results in a reduced population growth rate at 1 mg L^{-1} at standard test conditions which may indicate also consequences for the zooplankton community. In accordance, the populations break down at 1 mg L^{-1} after completing the first generation in the multigenerational study, at least at simulated spring conditions providing a temperature range of 11–27 °C. In contrast, the ecotoxicity of pyrimethanil appears to decrease with longer exposure time if comparing the NOEC values of the acute immobilization and chronic test with *C. riparius* (Table 2). It becomes obvious that EC_x values derived from acute response tests with *C. riparius* at either 20 °C or 18 °C–26 °C vary considerably (Table 2; Fig. 2) but this inter-experimental variation is within a normal range as shown by Weltje et al. (2010). The acute response test with *C. riparius* first instar larvae to pyrimethanil and temperature clearly shows that temperature modulates the toxicity of pyrimethanil at a concentration range of 0.5–32 mg L^{-1} (Fig. 2). Enhanced temperature stress often enforces the impact of toxic substances on aquatic organisms (Heugens et al., 2001, 2003; Holmstrup et al., 2010).

The increased acute ecotoxicity of pyrimethanil with increasing temperature (Fig. 2) might be explained by the enforced metabolism of midges or by the rising formation of toxic pyrimethanil metabolites with other modes of action than the mother substance. The formation of toxic metabolites is, however, not a probable cause for the enhanced toxicity because the known metabolites are far less toxic than the mother compound (DAR, 2005). Furthermore, the content of pyrimethanil in the medium should be stable during a period of at least seven days at 15 °C–25 °C (unpublished data; DT_{50} in the water phase = 16.5 days, EFSA, 2006). Hence a changed toxicokinetic profile with an altered absorption and elimination of the substance at different temperatures is a more probable explanation. Tests with Cd and daphnids have shown that an increased toxicity of the heavy metal is based on a better absorption of the substance in consequence of rising temperatures (Heugens et al., 2003, 2006). Moreover, Guerrero et al. (2002)

ascertained that the metabolism of fungicides and other xenobiotics to more polar products with a different mode of action is temperature-dependent. Up to now it is, however, not clear if either xenobiotics can be easier and faster taken up or if the accumulation in the organism becomes fortified at higher temperatures or if it is a combination of both effects (Heugens et al., 2003). How these effects can be traced back to changes in the metabolism of the *C. riparius* larvae, in terms of an easier pyrimethanil adsorption on the chironomid feeding tube, an exceeded pyrimethanil uptake or other modes of action of metabolites of the fungicide due to increased temperature has to be shown by further studies.

In contrast to the increased acute pyrimethanil ecotoxicity on *C. riparius* at higher temperature (Fig. 2), pyrimethanil at a previously determined NOEC level and three temperature scenarios do not act synergistically in *D. magna* (Fig. 3C). Indeed there seems to be a correlation of the total mortality and average reproduction with the mean temperature per scenario (Table 3). However, chronic effects of the low-concentration of pyrimethanil in *D. magna* under near-natural temperature regimes considering suboptimal, optimal and super-optimal temperatures reveals a complex, not inherently temperature-dependent reproductive pattern if considering the thermal history (Fig. 3C).

Reproduction of *D. magna* is significantly inhibited by pyrimethanil, but only if the controls are in an excellent reproductive shape as observed in few generations in the current and future warm year simulations (Table 3). It is a general rule, that the reproduction level increases linearly up to a physiological optimum as a result of an enhanced individual performance (Gabriel and Lynch, 1992), here in case of higher temperature. Concordantly the parent animals in the simulated scenarios 'warm year, today' and 'warm year, 2080' produce more juveniles and generations compared to the scenario 'cold year, today' over the fixed test period (Table 2). But the reproduction of *D. magna* declines after a first peak under the 'warm year, today' and 'warm year, 2080' scenarios and recovers despite still increasing temperature leading to a bimodal response pattern (Table 3). It is well known, that the reproduction rate declines if the reproductive (thermal) optimum is exceeded (Gabriel and Lynch, 1992). The observed bimodal pattern during the multigenerational study, however, is a phenomenon we only know from daphnid field populations in response to phytoplankton dynamics (Müller-Navarra and Lampert, 1996; Winder and Schindler, 2004).

Given that the bimodal response occurred at different time points in the two warm year simulations, simple experimental errors such as low food quality, irradiation effects or variance due to different experimentators can be excluded as an explanation for this unusual reproductive pattern. Probably the increased metabolism of the parent daphnids at higher temperature leads to a higher reproductive output, although energy resources still depend on the same food level over the test period. Then the reproduction rate of the juvenile daphnids should decline with further rising temperatures because food conditions for the next offspring become worse (Giebelhausen and Lampert, 2001; Straile 2002; Winder and Schindler, 2004). Nevertheless, the food was provided *ad libitum* during the whole experimental period. An alternative reason for the bimodal reproductive pattern might be that parent animals transmit information about current environmental conditions and variability to their offspring and these 'maternal' or 'epigenetic' effects cause phenotypic and genotypic changes in the juveniles and increasing the range of dynamics displayed by populations (Ginzburg and Taneyhill, 1994; Mousseau and Fox, 1998; Boersma et al., 1999; LaMontagne and McCauley, 2001). Especially abiotic factors like temperature, pH or photoperiod may affect directly (via maternal programming) or indirectly (via offspring sensitivity to maternally transmitted factors) progeny (Mousseau and Fox, 1998). LaMontagne and McCauley (2001) suggest that at least in daphnids a simple mismatch between the maternal and offspring environmental conditions is sufficient to produce dramatic phenotypically changes in offspring. Within the present study it might be the case that increasing temperature lead to an enhanced metabolism and egg production. The same case is true for the following generation but due to similar food amounts less food is available for the daphnids resulting in reduced progeny amounts. Amongst others a reduction in food can lead to a striking shift in energy allocation between growth and reproduction (LaMontagne and McCauley, 2001).

The bimodal reproductive pattern in the 'warm year scenarios' is diminished under additional pollutant stress. Only if control animals are in a phase of a production maximum, a toxic effect of pyrimethanil on reproduction became apparent. Moreover, the bimodal reproductive pattern of the daphnids is obvious only in the pyrimethanil treatments exposed to the simulation of a 'warm year, 2080'. A higher metabolic rate due to the combined impact of warm summer temperature and pyrimethanil exposure might influence the energy allocation at the expense of offspring production. As a consequence of enhanced metabolic rates under warmer conditions and exposure to pollutants, reduced energy resources should also be allocated for detoxification processes (Heugens et al., 2003; Antunes et al., 2004; Bossuyt and Janssen, 2004). The additional demand for detoxification processes may reduce the energy reserves of maternal animals which may no longer be sufficient to ensure a comparably high reproduction rate like in unexposed animals (Haeba et al., 2008).

For the eco(toxico)logical interpretation, the analysis of the *pgr* might be more relevant than single reproductive or survival endpoints and may allow conclusions about daphnid population dynamics in the field. This is due to the mere fact that the population growth for *Daphnia* species integrates their mortality, reproduction as well as their developmental rate which is not yet included in standard test protocols (Calow et al., 1997; Hammers-Wirtz and Ratte, 2000; Coors and De Meester, 2008). Compared to the study by Coors and De Meester (2008) similar results for the *pgr* were determined in the standard reproduction test. They figure out that pairs of stressors cause only minor reductions in *pgr* about 4–12%. These outcomes can be confirmed by our multi-generation study with decreases in *pgr* about 1.5–14.2% for all scenarios. There is only one exception under spring conditions for the 'future year, 2080' in the F_1-generation. Here, a decrease of about 30% for the *pgr* was calculated. This observation supports the assumption of Giebelhausen and Lampert (2001) that warming earlier in the growing season could have a more severe impact on daphnid performance than elevated temperatures in summer months. The *pgr* of daphnids is mainly determined by temperature gradients and only faintly by the low-dosed fungicide pyrimethanil, since negative pyrimethanil effects on mortality are buffered by an intensified reproduction. Therefore, *pgr* differs less among control and NOEC exposed populations within a given temperature scenario than between populations grown under different temperature scenarios.

Although the NOEC treatment caused only slight effects on population growth, the influence of pesticides for terrestrial and aquatic organisms and ecosystem should not be underestimated. In general it can be concluded that the higher the temperature is the more the *pgr* increases in all treatments and temperature scenarios (Fig. 4). Therefore we suppose, that climate change as simulated in our study may pose a low risk for the population growth of daphnids at optimal food conditions. From an ecological point of view it can be expected that daphnids reproduce earlier and more often in the year at thermal conditions forecasted for the global climate warming. Hence the consumption of algae by daphnids will become enforced. If the population dynamic of the phytoplankton

does not follow the same time lapse as suggested to occur under climate change by several authors, the basic food resource for the daphnids is missing. That may result in a decline of the zooplankton community and as an indirect effect also of secondary consumers like fish (Platt et al., 2003; Winder and Schindler, 2004).

5. Conclusion

Multigenerational experiments including endpoints at the population level can be regarded as a decisive point in the assessment of long-term effects of pollutants in the aquatic environment (Muyssen and Janssen, 2004). Although the reproduction inhibition of *D. magna* was ca. 20% on average and thus comparable as observed at the NOEC of the standard test at 20 °C, the chronic exposure to a LOEC (based on results of standard testing) prevents population growth of *D. magna*, whereas the chronic exposure of *D. magna* to a NOEC under near-natural temperature regimes over multiple generations reveals an inhibition of reproduction by up to 37%. These low-concentration pyrimethanil effects are only observed in the multigenerational study under near-natural conditions and cannot be deduced from standard tests. Thus ecological aspects, in particular thermal and multigenerational effects, should be considered if appraising the ecotoxicity of pesticides and assessing their ecotoxicological risk for the aquatic environment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.envpol.2012.04.020.

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