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Editor's choice

Effects of microcystin-producing and microcystin-free strains of *Planktothrix agardhii* on long-term population dynamics of *Daphnia magna*

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Abstract – The effects of cyanobacterial toxins on herbivorous zooplankton depend on cyanobacterial strains, zooplankton species and environmental conditions. To explore the relationship between zooplankton and cyanobacteria, we investigated the effects of *Planktothrix agardhii* extracts on *Daphnia magna* population dynamics. We designed an experiment where individuals were grown in the presence of extracts of two *P. agardhii* strains. We monitored daily life-history parameters of *D. magna* individuals subjected to microcystin-RR (MC-RR), intracellular and extracellular extracts of a microcystin-producing strain (MC-strain, PMC 75.02) and a microcystin-free strain (MC-free strain, PMC 87.02) of *P. agardhii*. Measured life-history parameters of *D. magna* were used to build population dynamics models and compute expected population growth rate, replacement rate, generation time and proportion of adult and juveniles at demographic equilibrium. Results show that MC-RR tends to slow the life history (reduced growth rate and larger proportion of adults). In contrast, intracellular extracts of the two strains tend to accelerate the life history (increased growth rate, decreased generation time and lower proportion of adults). Extracellular extracts produce the same trends as the intracellular extracts but to a lesser extent. However, the MC-strain has stronger effects than the MC-free strain. Interestingly, extracellular extracts of the MC-free strain may have effects comparable to pure MC-RR. Moreover, in the presence of MC-RR and both cyanobacterial extracts, the daily fecundities present a cyclic pattern. These results suggest that MC-RR and unknown metabolites of cyanobacterial extracts have negative effects on *D. magna* reproduction processes such as those observed with endocrine-disruptive molecules.

Key words: *Planktothrix agardhii* / *Daphnia magna* / *toxic cyanobacteria* / *population dynamics* / *matrix population model*

Introduction

The interaction between phytoplankton and herbivorous zooplankton is pivotal for aquatic ecosystem functioning as it mediates the transfer of energy and matter from autotrophs to heterotrophs. The ability of herbivorous zooplankton to handle and process phytoplankton is therefore of major importance as a lack of control of phytoplankton by their predators may lead to massive development of phytoplankton (Vanni, 1987; Vanni and Findlay, 1990; Hansson and Carpenter, 1993; Brett and Goldman, 1996). Phytoplankton may modify this control by developing defence strategies against herbivores. There are several kinds of defence strategies. Morphological defences include not only the formation of colonies and filaments that are difficult to handle by zooplankton (Burns, 1968) but also the development of spines or thorns (Lürling and Van Donk, 1997; Hamlaoui *et al.*, 1998; Lürling, 2003a). Chemical defences are the production of organic compounds that are toxic for grazers

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(Carmichael, 1992; Turner and Tester, 1997; Tillmann and John, 2002). Behavioural defences allow the prey to escape herbivory, either in space, by means of flagella or, in time, via resting stages (see references in Hansson *et al.*, 2007). All these defences affect ultimately the population dynamics of herbivorous zooplankton by modifying either the predator's functional response, its reproductive success, or its mortality.

Among phytoplankton, cyanobacteria are a bloom-forming group, well known for their toxic effects on humans and animals (for a review, see Codd *et al.*, 2005). As cyanobacterial blooms induce changes in water use (*i.e.* by rendering water unusable for domestic and agricultural use, recreation), they also represent an important alteration of ecosystem services when water of high quality is required. Such cyanobacterial dominance may be partly due to a weak control from large generalist zooplankton as suggested by negative relationship between cyanobacteria and cladocerans (Ghadouani *et al.*, 2006), such as *Daphnia* sp. and calanoid copepods (Hansson *et al.*, 2007) or between cyanobacteria and cyclopoid copepods (Catherine *et al.*, 2008). Indeed, the production of toxic metabolites is suspected to alter biotic communities through allelopathic interactions. Several hypotheses have been proposed concerning the role of such metabolites at several trophic levels (for reviews, see Vasconcelos and Pereira, 2001; Gross, 2003; Wiegand and Pflugmacher, 2005; Babica *et al.*, 2006; Leflaive and Ten-Hage, 2007; Schatz *et al.*, 2007). However, such a toxic activity is difficult to characterize because of (i) the presence of other molecules that may modify the effect of toxins (Rohrlack *et al.*, 1999b; Buryskova *et al.*, 2006), (ii) the adaptation of target organisms (Wilson and Hay, 2007) and (iii) the appearance of avoidance behaviour in some species of zooplankton (Kurmayer and Jüttner, 1999). As summarized above, several characteristics of cyanobacteria may contribute to lower zooplankton grazing pressure on cyanobacteria. Some cyanobacterial genera present large forms such as colonies or filaments that decrease zooplankton grazing (Webster and Peters, 1978). In addition, some genera produce organic compounds that may be toxic to zooplankton (DeMott *et al.*, 1991; Carmichael, 1992; Kirk and Gilbert, 1992; Ferrão-Filho *et al.*, 2000; Jang *et al.*, 2003). Cyanobacterial morphology, toxin production and their effects on zooplankton have stimulated numerous laboratory experiments and meta-analyses of these results show different things (Wilson *et al.*, 2006; Tillmanns *et al.*, 2008). The effects of cyanobacteria on grazer population growth rate depend partly on morphology as filamentous forms are better food than single-celled cyanobacteria. Surprisingly, the presence of known toxic compounds had no effects on (1) population growth rate in comparison with a diet without cyanobacteria and (2) survival rate in comparison with starvation, unless the *Microcystis* strain PCC7820 was included in the analysis and resulting in a negative effect on survival rate (Wilson *et al.*, 2006). Although food quality cannot be viewed directly as a defence, as the grazer should identify the prey before eating it, this first meta-analysis clearly showed that cyanobacteria are poor food by comparison to small chlorophytes and flagellates. In a second meta-analysis that investigates species-specific responses of zooplankton to cyanobacteria, Tillmanns *et al.* (2008) showed a large variation in these responses. In particular, there was no clear effect of cyanobacterial toxins on the growth of seven zooplankton species except for *Daphnia magna* Strauss whose population growth rate was negatively affected by cyanobacterial secondary compounds. Importantly, the two meta-analyses showed that most experiments investigating toxic effects of cyanobacteria focus on secondary metabolites known to be toxic. This implies that species (or strains) that do not produce these secondary metabolites are classified as non-toxic (Wilson *et al.*, 2006). The results of the two meta-analyses strongly suggest that the presence of undescribed toxins in experiments may blur their results.

Several experiments have shown that secondary metabolites other than microcystins (MC) may affect grazers' growth. Jungmann (1992) explored the effects of the MC-producing wild-type *Microcystis aeruginosa* PCC7806 on *Daphnia pulicaria* and showed that some compounds had a higher effect on *D. pulicaria* survival than pure MC-LR or [D-Asp^3]MC-LR. The same PCC7806 strain of *M. aeruginosa* and its *mcy*$^-$ mutant that does not produce MC were used to investigate the effects of MC on *Daphnia galeata* survival and ingestion rate. The results suggested that MC was the cause of *D. galeata* poisoning but ingestion inhibition was caused by other *Microcystis* compounds (Rohrlack *et al.*, 1999a). Rohrlack *et al.* (2004) showed more recently that the *M. aeruginosa* UWOCC CBS produces microvidin J, a toxin that induces lethal moulting disruption to *D. pulicaria*. Negative effects of a MC-free cyanobacteria, *Aphanizomenon flexuosum*, on egg production in *D. galeata* were also found; although this effect might be due to the nutritional quality of the cyanobacteria (Kurmayer, 2001). Finally, negative effects of a MC-free strain of *Planktothrix agardhii* were observed on *Thamnocephalus platyurus* (Keil *et al.*, 2002). All these results suggest strongly that other unidentified secondary metabolites may affect grazers' different life-history traits and at different stage of their lives, and consequently their population dynamics.

The aim of our experiment and analyses is to assess long-term effects of MC and secondary metabolites on population dynamics. These long-term effects are estimated by calculating demographic descriptors using matrix population models. To begin, we conducted a life-table experiment to assess the effects of a MC-producing strain (PMC 75.02), a MC-free strain (PMC 87.02) of *P. agardhii* (Yéprémian *et al.*, 2007) and pure microcystin-RR (MC-RR) on *D. magna* population dynamics. Strains of *P. agardhii* were isolated from a bloom in a lake from Base Nautique de Viry (BNV), in Paris area (France). In this lake, the dynamics of *P. agardhii* populations was controlled by several environmental factors including zooplankton grazing (Catherine *et al.*, 2008). It is also well known that the cell concentration in MC varies with

cyanobacterial population growth (Park *et al.*, 1998; Lyck, 2004; Jang *et al.*, 2008) and this might also be the case of other secondary metabolites. Moreover, MC are stored in cells and released in the medium with cell lysis (Park *et al.*, 1998; Rohrlack and Hyenstrand, 2007) and their concentration in water and in biomass may display a seasonal dynamics (Pawlik-Skowronska *et al.*, 2008). Other secondary compounds might have the same fate and also be actively exported. Therefore, we subjected *D. magna* individuals to intracellular or extracellular extracts of the two *P. agardhii* strains culture in exponential growth or in plateau phase. In general, experiments exploring toxic effects of cyanobacteria compared life-history traits (fecundity and mortality rate) under different expositions. However, these measures do not allow the assessment of long-term population dynamics. Using matrix population models, we could compute demographic descriptors (growth rate, generation time, replacement rate and percentage of juveniles and adults) of *D. magna* populations at demographic equilibrium and compare population characteristics under different expositions with *P. agardhii* extracts or MC-RR.

Methods

Experimental organisms

D. magna clones were isolated from a pond situated in the Ecole Normale Supérieure, Paris, France, where no blooms of cyanobacteria have been observed. Clones were cultivated in an aquarium (20 L filled up to a third with Volvic© water) at 22 ± 1 °C with a 14 h light:10 h dark cycle, and fed *ad libitum* with *Scenedesmus obliquus* (CCAP 276/6A) cultured in COMBO medium (Kilham *et al.*, 1998).

Experiments were performed with two monoclonal and non-axenic strains of *P. agardhii* isolated from BNV, France (Yéprémian *et al.*, 2007) and maintained in the Paris Museum Collection (PMC). The *P. agardhii* strain PMC 75.02 produces mainly three variants of MC (*m*/*z* 981.6 [D-Asp^3]MC-LR; *m*/*z* 1024.8 [D-Asp^3]MC-RR; and *m*/*z* 1045.6 [D-Asp^3]MC-HtyR). The *P. agardhii* strain PMC 87.02 is a MC-free strain (Yéprémian *et al.*, 2007). The strains are hereafter referred as MC-strain and MC-free strain, respectively. Each strain was cultured in four modified 2 L Duran bottles containing 1 L of Z8 medium (Kotai, 1972) and inoculated with 100 mL of an active culture. The bottles were placed in growth chambers at 20 ± 1 °C, and illuminated with cool white fluorescent tubes (Osram lumilux Plus Eco, L18W/21-840) with a 16 h light:8 h dark cycle, under 10 ± 2 µmoles photons.m^{-2}.s^{-1} (Yéprémian *et al.*, 2007). Cultures growth was monitored by measuring optical density at 436 and 750 nm every 2 days with a spectrophotometer (Foy, 1980). At late exponential growth phase, that is 22 days for the MC-strain and 14 days for the MC-free strain, 0.5 L were sampled in each bottle and pooled to obtain 2 L of cultures at exponential phase of each strain. At 41 days for the MC-strain and 40 days for the MC-free strain, the remaining 0.5 L of each culture were pooled to obtain 2 L of cultures at plateau phase of each strain. MC concentration was measured with a protein phosphatase 2A (PP2A) inhibition assay as described in Yéprémian *et al.* (2007). The MC concentrations of the MC-strain in the stock cultures were 225 µg eq. MC-LR.L^{-1} in exponential phase (eq. to 148×10^{-9} µg eq. MC-LR per cell) and 500 µg eq. MC-LR.L^{-1} in plateau phase (eq. to 23×10^{-9} µg eq. MC-LR per cell). All the cultures were then filtered by parts of 20 mL with GF/C filters. Filters, which retained intracellular contents of cyanobacterial cells, and filtrates containing cyanobacterial exudates, were then kept at -80 °C. Extracts filters were obtained after grinding with ultrasonication in 7.5 mL of osmotic water in centrifugation tubs. The tubs were then centrifugated at 3000 ***g*** and 10 °C for 10 min. The supernatant was filtered with a GF/C filter and gave the intracellular extracts.

Experimental design

The aim of the experiment is to determine the life-history parameters (survival and fecundity) of populations, *i.e.* groups of individuals, subjected to different treatments in order to compute demographic descriptors and perform demographic analysis. In this context, the experiment amounts to study independent populations of 15 individuals at day 0 (see below). The populations are defined by the treatment they had undergone. Ten treatments were applied: control, addition of pure MC-RR (Alexis® Biochemicals) at a final concentration of 3 µg.L^{-1} in the Daphnia medium, and addition of intra or extracellular extracts from the exponential or plateau phase of the MC-strain or MC-free strain (8 extract treatments). To compare toxic effects of cyanobacterial extracts with those of pure MC, we chose MC-RR whose toxicity is different from those of MC-LR (LD50 600 and 50 on mouse, respectively, Sivonen and Jones, 1999) and mimic an exposure to a less toxic MC such as the demethyl forms (*e.g.* LD_{50} of [D-Asp^3]MC-LR ranged from 160 to 300 on mouse). Prior to the experiment, respectively, for the first, second and third rounds, 45 and 60 individuals from our *D. magna* culture were isolated to obtain neonates after one generation. This experimental design, which includes genetic diversity in populations, was the same for all treatments.

At day 0, 15 neonates (< 24 h) per treatment were individually placed in mineral water in 40 mL jar in the same conditions as *D. magna* cultures. Neonates came from at least 15 different litters so that individuals from each litter could be assigned to each treatment. Individuals were fed daily with *S. obliquus* (CCAP 276/6A) at a concentration of 0.1 mg C per individual until the age of 3 days and at a concentration of 0.2 mg C per individual thereafter. When algae were added, the corresponding volume of medium was removed. Each day, all individuals were checked for survival and offspring number. Offspring were counted and removed every day. The experiment

lasted 40 days and treatments were repeated every day. As the different treatments amount to observe ten independent populations, the treatments were conducted in three independent rounds: (1) treatments of pure MC-RR, of intracellular or extracellular extracts of the MC-strain in exponential growth phase; (2) control and treatments of intracellular or extracellular extracts of the MC-strain in its plateau phase; and (3) treatments of intracellular or extracellular extracts of the MC-free strain in its exponential growth and plateau phases. The concentration of MC-RR was chosen to equal the mean concentration in MC-LR eq. found in the BNV (France) during the permanent bloom (Briand *et al.*, 2002) when outliers (peak concentrations) are excluded. The concentration of intracellular extracts of the exponential and plateau phases of the MC-strain that were added each day to each individual was calculated so that the total volume (intracellular extract + extracellular extracts) reaches the final concentration of 3 $\mu g.L^{-1}$. The concentration of intracellular extracts of the exponential and plateau phases of the MC-free strain that were added to each individual was calculated to reach the same cell density as in the intracellular extracts of the exponential and plateau phases of the MC-strain. The computation of the volume of extracellular extract from the exponential and plateau phases of the MC and the MC-free strains added to each individual was the same in the four treatments. It corresponded to the total volume of *P. agardhii* culture diluted to a final concentration of 3 µg MC-LR eq. L^{-1} minus the volume of intracellular extracts. As pure MC-RR was diluted in methanol, we added 1.2 µL of methanol at 80% in all treatments except for pure MC-RR to obtain an equivalent methanol final concentration (0.003%, v/v).

Demographic analysis

The demographic analysis relies on an accurate description of the life-cycle of *D. magna* and its corresponding matrix model. After defining a time step, the life-cycle is represented by a directed graph: the nodes describe the various stages the organism goes through during its life, and the arrows bear coefficients describing the contribution of a stage to another during a time step (Ferrière *et al.*, 1996). These coefficients correspond to the vital rates (survival and fecundity) that the experimental design described above allowed us to estimate. In the case of *D. magna*, in our experiment, the production of neonates by parthenogenesis occurs about every other day. We therefore took a time step of one day. After hatching, there are immature stages. Then females start to reproduce, thus defining the age at first reproduction α. The age-specific fecundity f_i is the product of litter size by litter success, where the subscript i refers to age. Individuals move each day from an age class to the next with an age-specific probability of surviving s_i. Our experiments lasted 40 days but some individuals were still alive at the end. The probability of surviving at age 40 was therefore set to 0. As no males were produced by females during our experiments, densities refer to female only (Fig. 1).

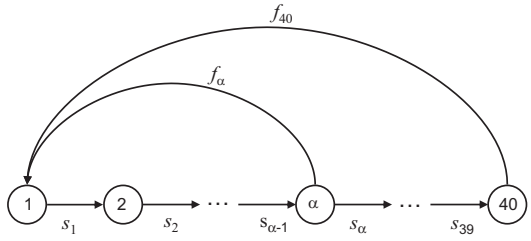

Fig. 1. Life-cycle graph of *D. magna*. The nodes represent the different stages, the arrows the contribution of one stage to another, s_i stands for survival rate, f_i for fecundity and α is the age at first reproduction.

The life-cycle translates into the transition matrix:

$$A = \begin{bmatrix} 0 & \dots & 0 & f_\alpha & \dots & f_{39} & f_{40} \\ s_1 & \dots & 0 & 0 & \dots & 0 & 0 \\ & \ddots & & & & & \\ & & s_{\alpha-1} & & & & \\ 0 & \dots & & s_\alpha & \dots & 0 & 0 \\ & & & & \ddots & & \\ 0 & \dots & 0 & 0 & \dots & s_{39} & 0 \end{bmatrix}.$$

The matrix $A = (a_{ij})$ summarizes all contributions from one stage to another and allows to describe population changes from time t to time $t+1$ (a_{ij} is the time-average contribution of an individual in class i at time t to class j at time $t+1$). We denote $n(t) = (n_1(t), n_2(t),\ldots, n_{40}(t))$ the (column) population vector whose entries are the number of individuals in each age class at time t. The population vector is updated from a time step to the next according to

$$n(t+1) = An(t).$$

In this model, the population reaches a state of exponential growth with rate λ. The long-term growth rate λ is the dominant eigenvalue of the matrix (Caswell, 1989). Concomitantly, the population enters the stable age distribution, where the proportion of individuals in each age class becomes constant.

In the following, we will discuss for each treatment the corresponding population growth rate λ, the net-reproductive rate R_0 (hereafter called *replacement rate*) which is "the expected number of offspring by which a newborn individual will be replaced by the end of its life" (Caswell, 1989), and $\bar{T}$ the generation time defined as the mean age of the mothers of the offspring produced by a population at the stable age distribution (Caswell, 1989). From the stable age distribution, we computed the proportion of reproductive and non-reproductive individuals in the population.

Daily fecundity rates were estimated by averaging the number of one-day-old juveniles produced per female each day. In the experiments we followed a cohort, so that the daily survival rates could be estimated by the ratio of the number of individuals alive on a given day to the number of individuals alive the previous day. A population matrix

Table 1. Effects of the treatments on population dynamics of *D. magna*. The treatments are: MC-RR: pure MC-RR; *P. agardhii* extracts are represented by MC or MC-free for MC or MC-free strains. The code for the treatments is: first letter E or P for exponential or plateau phase and, second letter, E or I for extra or intracellular extracts. Population parameters are: λ, population growth rate; R_0, replacement rate, NR/R, percentage of non-reproducer/reproducer, and $\bar{T}$, generation time.

	Day of first clutch (day)	Mean fecundity	Mean survival rate	λ (day^{-1})	R_0 (ind)	NR/R (−)	$\bar{T}$ (day)
Control	7	7.43	0.99	1.42	211	88.1/11.9	9.70
MC-RR	7	4.26	0.98	1.29	73	80.7/19.3	11.33
MC-E-E	7	8.61	0.97	1.43	142	90/10	10.05
MC-E-I	6	10.19	0.99	1.62	296	90.9/9.1	8.40
MC-P-E	7	7.08	0.99	1.41	179	87.4/12.6	10.59
MC-P-I	6	12.33	1.00	1.62	401	91.5/8.5	8.22
MC-free-E-E	8	9.97	0.99	1.44	254	93.2/6.8	10.80
MC-free-E-I	7	7.24	1.00	1.54	246	92.6/7.4	9.48
MC-free-P-E	9	4.72	0.99	1.28	128	87.8/12.2	15.09
MC-free-P-I	7	6.07	0.99	1.51	170	92.1/7.9	9.48

was thus defined for each treatment. Demographic descriptors — growth rate λ, replacement rate R_0, generation time $\bar{T}$, proportion of reproductive and non-reproductive individuals — were computed using the Unified Life Models (ULM) software (Legendre and Clobert, 1995). We also calculated for each treatment, the mean fecundity and the mean survival rate as the arithmetic mean of the fecundities of reproducers and the geometric mean of survival rates, respectively.

Statistical analysis

We used R (version 2.8.0) for all statistical analyses and graphs. We tested for a relationship between mean fecundity and mean survival rate with a linear model. The *P*-value for significance was set *a priori* at 0.05. As age-specific fecundities appeared to display some periodicity, we analysed them using the spectrum function of R. Note that each set of computed demographic descriptors describes one independent population. As one treatment corresponds to one population, we could not estimate variability and therefore we could not assess the significance of the differences between populations' demographic descriptors.

Results

Before analysing results, one should keep in mind that the aim of the study is to make population projections, that is, to describe the population dynamics if the experimental conditions were to remain constant (Caswell, 1989). With the life-history parameters obtained from the experiments, we could simulate population dynamics on the basis of individual responses to the treatments. We present here characteristic variables of the simulated populations if individuals were maintained under the same environmental conditions. These demographic descriptors computed from the life-cycle account for genotypic and phenotypic characteristics of an average individual within the population, in a given environment. They allow to describe the various responses of the organism to the different treatments, and to infer the biological mechanisms underlying these responses.

The mean survival rate is not affected by the different treatments: its value ranges from 0.97 to 1 (Table 1, Fig. 2(a)). The mean fecundity changes more drastically in the different treatments: it ranges from 4.26 in the treatment pure MC-RR to 7.43 in the control and 12.33 for individuals subjected to intracellular extracts of the MC-strain in plateau growth (Table 1, Figs. 2(a) and (b)). Intracellular extracts from the MC-strain induce the highest fecundities, while intracellular extracts of the MC-free strain induce fecundities close to those of the control. Fecundities in extracellular extract treatment are also close to the control, except for extracellular extracts from the MC-free strain at plateau phase (4.72) where the mean fecundity is close to the one with pure MC-RR (Table 1, Figs. 2(a) and (b)). There is no significant relationship between mean fecundity and survival rate ($P > 0.05$).

The comparison of the treatment with pure MC-RR and the control show that, in the treatment with pure MC-RR, the population growth rate is lower (1.29 day^{-1} *versus* 1.42 day^{-1}) and the replacement rate is about one-third of the replacement rate in the control (73 *versus* 211), which results in a higher percentage of reproducers in the population (Table 1). The generation time is higher in the treatment with pure MC-RR than in the control (Table 1). In the two treatments, age at first reproduction was 7 days.

The examination of population growth rates obtained from individuals subjected to the two *P. agardhii* extracts shows that main differences come from the strains but the effects of intra and extracellular extracts display the same trends for the two strains (Table 1, Fig. 2(c)). For the MC and MC-free strains, the population growth rate is higher with intracellular extracts (*ca.* 1.6 and 1.5 for the MC and MC-free strains, respectively) than with extracellular extracts (*ca.* 1.4 for both strains and 1.3 for MC-free strain in exponential growth). The population subjected to extracellular extracts from the plateau phase of the

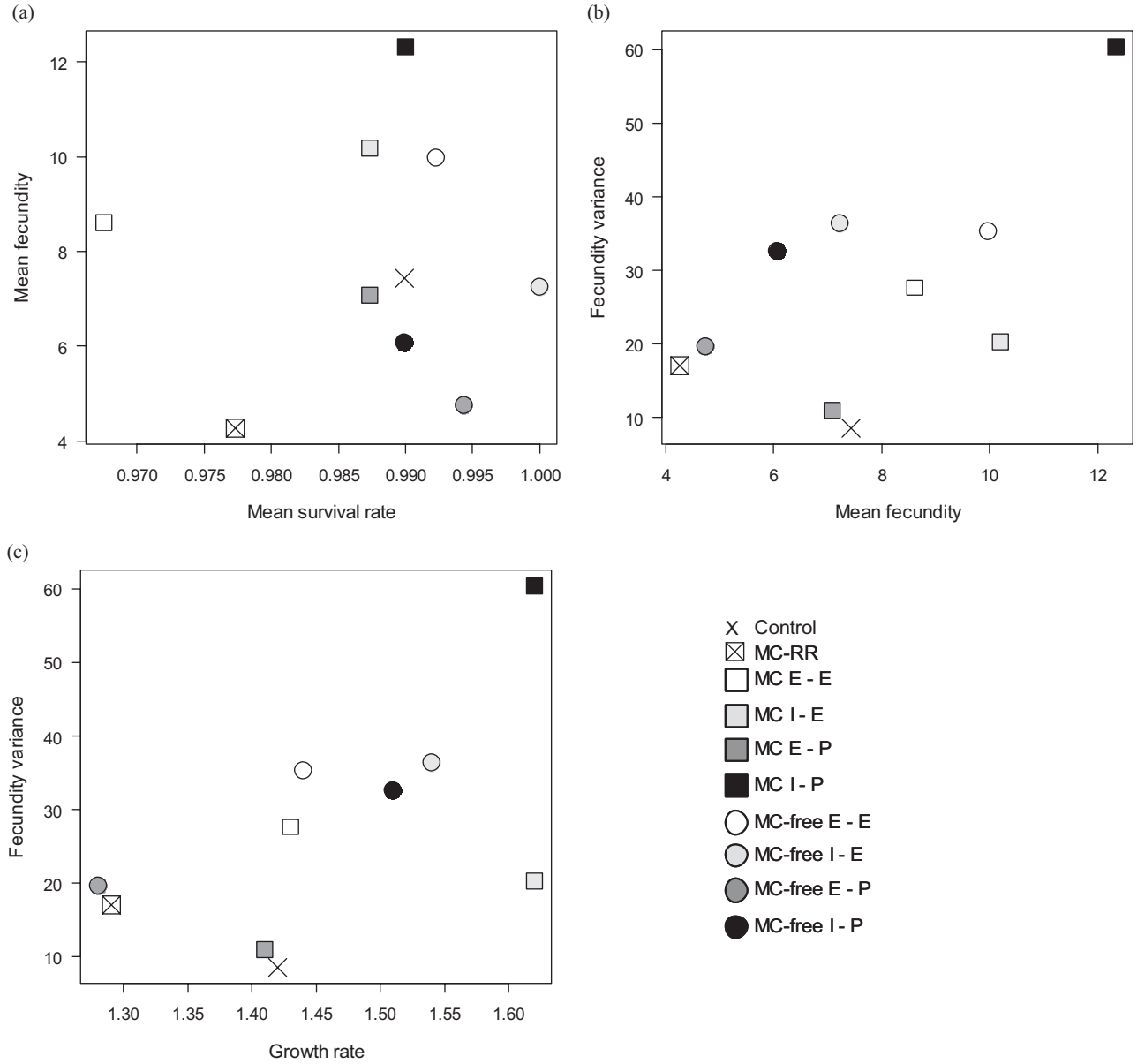

Fig. 2. Parameter descriptors. (a) Mean fecundity as a function of mean survival rate. Mean fecundity is the mean number of 1-day-old juveniles produced per female each day (ind.day^{-1}). Mean survival rate is the geometric mean of survival rates (day^{-1}). (b, c) Variance of the fecundity as a function of (b) mean fecundity and (c) the growth rate. The different treatments are represented as follows. Control: X; MC-RR: S. ⊠ *P. agardhii* extracts are represented by MC or MC-free strains. The code for the treatments is: first letter E or P for exponential or plateau phase and, second letter, E or I for extra or intracellular extracts.

MC-free strain has a growth rate comparable to one of the population subjected to pure MC-RR although the strain does not produce MC (Table 1). Replacement rates do not show the same trends. The populations subjected to intracellular extracts of the MC-strain have the highest replacement rates (296 and 401 individuals) with a first clutch at 6 days, whereas populations subjected to extracellular extracts of the same strain have replacement rates smaller than the control replacement rate (142 and 179 individuals *versus* 211 individuals) with a first clutch at 7 days (Table 1). For the MC-free strain, intra and extracellular extracts from the exponential growth phase induce replacement rates higher than the control (254 and 246 individuals *versus* 211 individuals), while extracts from the plateau phase induce replacement rates below the control (128 and 170 individuals). First clutch occurs at 7 days with intracellular extracts of the MC-free strain, while it is postponed at 8 and 9 days with extracellular extracts from the exponential and plateau phase, respectively. We observed in this latter treatment the production of neonates that sank to the bottom in 3 out of the 15 jars between days 9 to 12 (F. Hulot, personal observation). Note that the replacement rate with the extracellular extracts from the MC-free strain plateau phase is again closer to the pure MC-RR treatment than the control (Table 1).

The analysis of the stable-age distribution shows that populations subjected to extracellular extracts from the plateau phase of the two strains have a higher proportion of reproducers (12.6 and 12.2%) than the control population (11.9%), whereas populations subjected to other extracts (intracellular extracts and extracellular extracts from the exponential growth of the two strains) have a smaller proportion of reproducers (between 6.8 and 10%) than the control population. However, these populations are much "younger" than the population subjected to pure MC-RR (19.3%), which is also the less productive one and with the lower replacement rate (Table 1). The generation time $\bar{T}$ is slightly lower for populations subjected to intracellular extracts (between 8.22 and 9.48 days) than the control population (9.70 days) (Table 1). Except for the population subjected to extracellular extracts from the plateau phase of the MC-free strain, which has the highest generation time (15.09 days), the $\bar{T}$ of the populations subjected to extracellular extracts are very close (10.05–10.80 days) and intermediate to the $\bar{T}$ of the control and the pure MC-RR treatment.

Figures 2(b) and (c) summarizes the impact of the treatments on *D. magna* fecundity. Pure MC-RR induces a lower fecundity than the control. Overall, the MC- strain induces a higher fecundity than the control, except for the treatment with extracellular extracts from plateau phase, while the MC-free strain induces a lower fecundity than the control, except for the treatment with extracellular extracts from exponential growth phase. Both strains induce a larger variance in fecundity than the control. The representation of our results with the variance in fecundity as a function of the growth rate λ (Fig. 2(c)) – a descriptor integrating all demographic parameters (daily fecundity and survival rates, age at first reproduction) – shows demographic effects of the treatments that do not appear in Figure 2(b). For example, despite a lower mean fecundity than the control, the intracellular extracts from the plateau phase of the MC-free strain induces a larger growth rate than the control. This is due to a shorter generation time (Table 1). There are large deviations across specific cases (extracellular *versus* intracellular extracts; exponential *versus* plateau phase), showing the pleiotropic effects of *P. agardhii* on *D. magna*.

Daily fecundities correspond to a period of 3 days in discrete scale in all treatments except for the control and the extracellular extracts from the plateau phase of the MC-strain (Figs. 3(a), (c) and (e)). This periodicity is confirmed by the periodograms, which show a frequency of 0.36 per day for all treatments except for the two mentioned above (Figs. 3(b), (d) and (f)). This frequency corresponds to a period of 2.8 days, which is in agreement with the 3-day period in discrete time scale.

Discussion

The aim of our experiments was to assess the effects of known and unknown cyanobacterial compounds, including several variants of MC, on *D. magna* demography. Experiments were therefore designed to rule out nutritional deficiency of cyanobacteria and grazing resistance with morphological features as *D. magna* individuals fed with *S. obliquus* and only crude extracts of two strains *P. agardhii* were added. Our results show that, despite the absence of obvious effects of the treatments on mean fecundity and mean survival rate, *D. magna* population dynamics are differently affected according to the origin of the cyanobacterial extracts (strains or growth phase) or pure toxin. In comparison with the control, despite genetic diversity, a population exposed to pure MC-RR has a lower growth rate, a lower replacement rate, a lower percentage of juveniles and has a cyclic reproduction. Exposition of *D. magna* to one or the other strain of *P. agardhii* thus induces different population dynamics.

Effects of the MC-producing strain of *P. agardhii* (PMC 75.02)

When exposed to extracellular extracts of the M-strain, which includes compounds that would be naturally excreted, the *D. magna* population dynamics are close to the control population whatever the phase growth of the strain culture. However, when exposed to intracellular extracts of the MC-strain, which are compounds that are consumed by the herbivore, the population has a higher growth rate, a higher replacement rate and a higher percentage of juveniles. Whatever the two growth phase (exponential and plateau) extracts of *P. agardhii* culture, no major differences were observed in the reproduction schedule, both displaying a 3-day cycle. The absence of negative effects of the MC-strain of *P. agardhii* is not surprising as a meta-analysis has shown that the presence of common toxic compounds has no overall effect on herbivore population growth rate (Wilson *et al.*, 2006). The effects are rather cyanobacteria species-specific and, based on a second meta-analysis (Tillmanns *et al.*, 2008), we could expect a negative effect that only reduces the population growth rate without compromising exponential growth. In our study, the highest growth rate is recorded for *D. magna* populations subjected to intracellular extracts of the MC-strain. As the individuals were fed *ad libitum*, they were not in a nutritionally deficient state, but intracellular extracts were additional food that did not require handling. However, we cannot rule out positive effects due to compounds that stimulated *D. magna* fecundity. The two processes are not mutually exclusive and both compensate the potentially negative effects of MC. In a precedent work, we showed that in a 2-year survey of natural population of *P. agardhii* (Catherine *et al.*, 2008), there is no clear relationships between cladoceran abundance and *P. agardhii* biomass. Yet we demonstrated a negative relation between cyclopoids and *P. agardhii* dynamics related to a decrease of *P. agardhii* biomass that may have allowed zooplankton grazing activity to become more efficient. Furthermore, during the spring and summer of the survey, we observed an

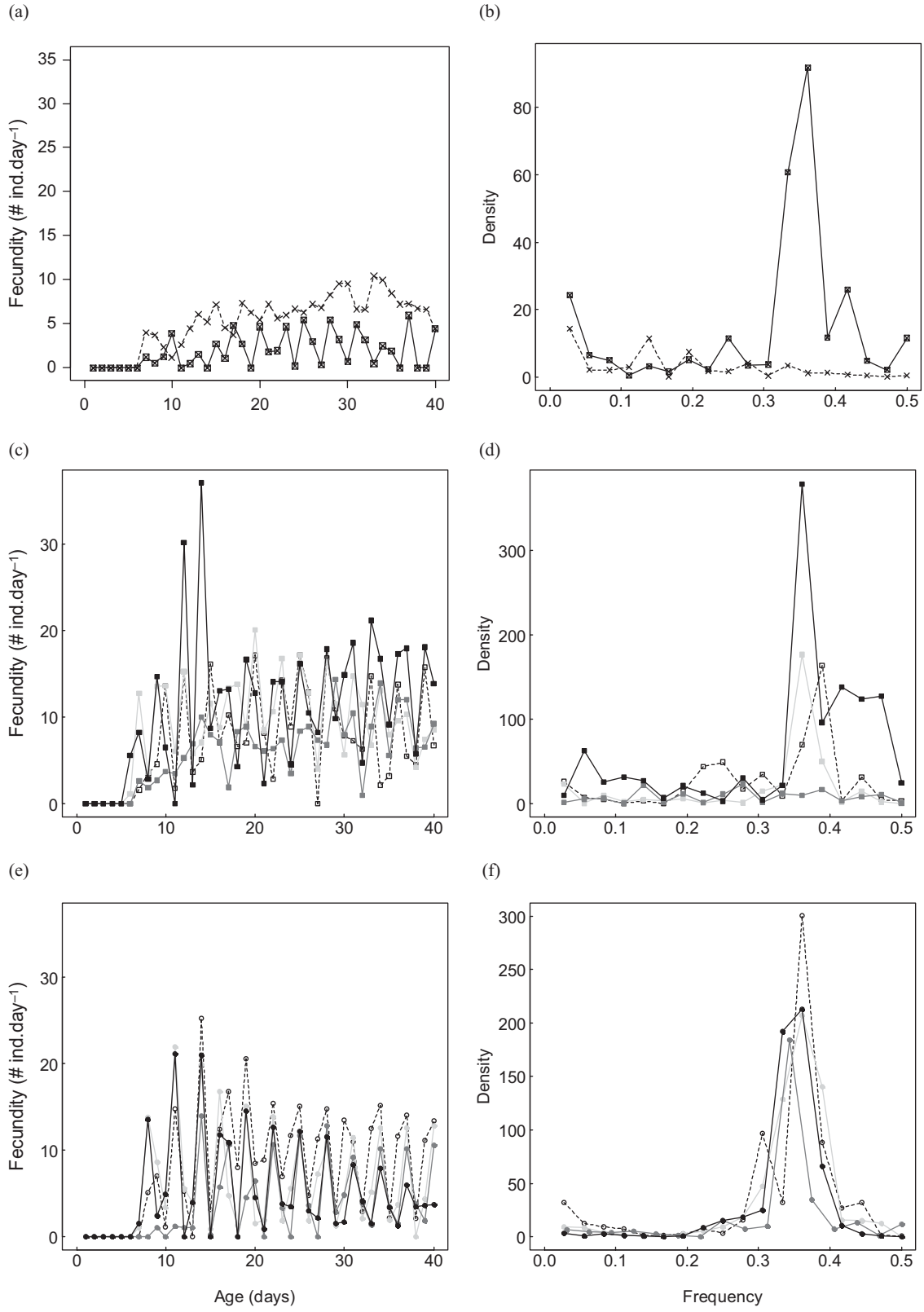

Fig. 3. Fecundity schedule and associated periodogram. Mean fecundity as a function of the age of individuals in (a) the control population and the population submitted to pure MC-RR, (c) the population submitted to extracts of the MC-strain, and (e) the population submitted to extracts of the MC-free strain. (b, d and f) Corresponding spectral density of the daily fecundities (periodogram). A peak in the periodogram gives the main frequency of the fecundity schedule if any. Treatments are designated as in Figure 2.

increase in zooplankton abundance. In the light of this study, we can hypothesize that it is the consequence of fecundity stimulation by *P. agardhii* populations in relation to a more efficient grazing and/or to a release of extracellular compounds irrespective of the toxin production.

Effects of the MC-non-producing strain of *P. agardhii* (PMC 87.02)

The same trends are found to a lesser extent with intracellular extracts of the MC-free strain. Either the additional resources provided by the extracts were less nutritive than those of the MC-strain or the stimulating effect on *D. magna* fecundity was less important. Conversely to the MC-strain, results show striking differences between the extracellular extracts of the MC-free strain from the exponential and plateau phases. The former shows the same trend than its MC-strain counterpart but the later differs markedly. Indeed, with these extracts, the population growth rate and the replacement rates are close to those of populations subjected to pure MC-RR. The first reproduction occurs at 9 days and this induces the highest generation time. Strong negative effects of the extracellular extracts from the plateau phase of the MC-free strain are therefore recorded although this strain does not produce known toxins. This result thus suggests the existence of unknown toxic compound(s) produced by the MC-free strain PMC 87.02 of *P. agardhii* affecting *D. magna* population dynamics through a delay in first reproduction. These compounds are predominantly produced during the plateau phase of *P. agardhii* PMC 87.02 growth and seem to be actively excreted in the medium. The effects of unknown toxins (or new to science) have been described with *P. agardhii* (Keil *et al.*, 2002), *M. aeruginosa* UWOCC CBS (Rohrlack, *et al.*, 2004), *M. aeruginosa* PCC 7806 (Jungmann, 1992; Lürling, 2003b) and *A. flexuosum* (Kurmayer, 2001), and are most probably widespread. Further investigations on the metabolite composition of the extracellular medium are needed. We have to keep in mind that the two strains PMC 75.02 and 87.02 may have more genomic differences than the known one of *mcy*B gene (Yéprémian *et al.*, 2007), and thus may also produce different metabolites. It is well known that the diversity of metabolites is very large within *P. agardhii* populations and/or strains. For example, 11 different MC chemotypes were identified from isolated clones in the monospecific population of *P. agardhii* from the BNV.

MC-RR and *P. agardhii* extracts as reproduction function-disruptors

The addition of pure MC-RR also caused a cyclic fecundity pattern in *D. magna*: a peak in the number of juveniles produced was found every 3 days. This 3-day cyclic pattern in the fecundity schedule was also observed with *P. agardhii* extracts, irrespective of the strain. Moreover, the daily fecundities appear in phase, *i.e.* the peaks of fecundity occur at the same time. This result, together with the negative effect on fecundity, suggests that MC-RR have a strong effect on the reproduction pathways. Reproductive functions are regulated by hormones and are consequently subjected to disruption by naturally occurring compounds acting as oestrogens. Among these contaminants, toxins may act as endocrine-disruptors, which modify the functioning of the endocrine system and induce, among other effects, reproductive disorders (Burkhardt-Holm, 2010). Such endocrine-disrupting effects of two cyanobacterial toxins, nodularin-R and MC-LR, have been observed *in vitro* by Oziol and Bouaïcha (2010) using a transgenic human cell line MELN. The authors inferred the existence of an indirect interaction of MC-LR with oestrogen receptors. We show here a similar effect with MC-RR on *D. magna*. In the same experimental conditions, we also observed the production of ephippia by isolated *D. magna* individuals subjected to MC-RR (Müller *et al.*, unpublished data), which supports the hypothesis of MC-RR acting as a reproduction disruptor. This cyclic production of juveniles was also observed with cyanobacterial extracts from the MC-strain PMC 75.02 and the MC-free strain PMC 87.02 of *P. agardhii*. Moreover, the age at first reproduction was delayed by the addition of MC-free strain extracellular extracts. These results suggest that the two strains of *P. agardhii* produce compounds that have reproduction disruptive effects with differences between the two strains in the compounds produced and their effects. Comparable toxicological effects have been observed with pesticide clearly defined as endocrine disruptor on *D. magna*. Chronic exposure of neonates (< 24 h old) for 21 days to the pesticide cypermethrin increased time to first brood (from 7 to 8–9 days) (Kim *et al.*, 2008). In an experiment exploring the effects of the two hormones testosterone and 4-hydroxyandrostenedione (4-OHA) on *D. magna*, Barbosa *et al.* (2008) showed endocrine disruptive effects of the two compounds. Testosterone decreased fecundity with an increase in aborted eggs and 4-OHA increased mortality of the neonates. The authors studied the effects of the two hormones on the fecundity of the first four broods. Interestingly, the results displayed in their Figure 2 show irregularities in the fecundities. For nominal testosterone concentrations from 0.15 to 0.62 $mg.L^{-1}$, the fecundity increases from the first to the fourth brood. However, for nominal testosterone concentrations of 1.24 and 2.48 $mg.L^{-1}$, the fecundity increases from the first brood to the second, then decreases for the third brood, and then increases again for the fourth brood with values higher than those of the second brood (Barbosa *et al.*, 2008). This pattern reminds the 3 days cyclic pattern found in our experiment with MC-RR and *P. agardhii* extracts. Our results show that MC-RR and the two strains of *P. agardhii* extracts have endocrine disruptive effects comparable to testosterone on *D. magna*. We can then hypothesize that MC-RR or *P. agardhii* extracts could mimic or antagonize the effects of hormonally active

agents involved in the reproduction processes. In addition, the MC-free strain produces compounds that delay age at first reproduction.

Conclusion

A demographic approach allows inferring the population level consequences of processes affecting individual – here the impact of two strains of phytoplankton on *D. magna*. The two strains PMC 75.02 and 87.02 of *P. agardhii* have different effects on *D. magna* and these differences are not solely due to the production of MC. The MC-free strain PMC 87.02 also affects *D. magna*, which suggests *P. agardhii* may produce other, unknown compounds that have negative biological effects. Our results suggest that MC-RR and *P. agardhii* extracts have reproduction function-disruptive effects on a Cladoceran, *D. magna*. These results call for long-term studies on the effects of cyanobacterial compounds in relation to the reproductive success of zooplankton.

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