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Feasibility of planctomycetes as a nutritional or supplementary food source for *Daphnia* spp

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Abstract – *Daphnia magna* is widely used as a standard organism in ecotoxicology assays. It plays a key role in energy transfer in freshwater food webs as a primary consumer, grazing on microalgae, yeast and bacteria. Daphnids are commonly reared in the laboratory using microalgae cultures but alternative or complementary sources are important to reduce the dependency on a single food source. The role played in nature by planctomycetes as a food source for other higher trophic levels is still unknown. In this study, we aimed to evaluate the potential of *Rhodopirellula rubra* strain LF2 as a nutritional or a supplementary food source for *D. magna* and *Daphnia longispina*. Life-history assays were conducted with daphnids fed with *R. rubra* in exponential and stationary growth phases, in three concentrations. Additionally, its adequacy as a supplement to the microalga *Raphidocelis subcapitata* was tested. In general, both daphnids showed impairment in all the parameters evaluated, especially when fed with *R. rubra*. However, when daphnids were fed with the two food sources, no changes were recorded for the rate of population increase. At the tested concentrations, *R. rubra* was not a good alternative food source in the daphnid diet.

Key words: Nutritional food / *Daphnia magna* / *Daphnia longispina* / *Rhodopirellula rubra* / life-history

Introduction

Daphnia is a genus of freshwater planktonic crustaceans that belong to the class Branchiopoda and the order Anomopoda (Alonso, 1996). Daphnids play a key role in energy transfer in freshwater food webs as a primary consumer (filter feeder) and controller of phytoplankton biomass in lentic ecosystems. These small crustaceans, commonly denominated water fleas, are able to control microalgal blooms but are the preferred food item of zooplanktivorous fishes (Rinke and Vijverberg, 2005), which causes large fluctuations in density and limits their growth season. *Daphnia* are largely non-selective filter feeders that do not discriminate between food particles with regard to their nutritional quality, grazing on microalgae, yeast, bacteria and protozoans (DeMott, 1989; Antunes *et al.*, 2003). Indeed, *Daphnia* spp. efficiently consumes heterotrophic bacteria (Gophen and Geller, 1984; Brendelberger, 1991; Pace and Cole, 1994), and are therefore able to shape the bacterial community structure and to suppress bacterial biomass production (Jurgens, 1994; Langenheder and Jurgens, 2001; Degans *et al.*, 2002).

Water fleas have become models in ecology, evolution and ecotoxicology, given their modest maintenance requirements, short life cycle, high fecundity and parthenogenetic (clonal) reproduction. *Daphnia* reproduces asexually under optimal conditions, and the first reproductive event is usually observed around 8–10 days after birth, with new broods being produced every 3–4 days (Ebert, 2005). *Daphnia* are commonly reared in the laboratory using unialgal cultures as a single food source (*e.g.,* Antunes *et al.*, 2004; Bukovinszky *et al.*, 2012; Meng *et al.*, 2014). Because they are fed with a single carbon source, culture performance becomes excessively dependent on the food source (microalga), which sometimes leads to fluctuations in survivability and reproductive output and occasional culture crashes (Baird *et al.*, 1989b; Sterner *et al.*, 1993). It is therefore important to find carbon sources that may supplement and diversify

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their diet (for example, yeast – see Loureiro *et al.*, 2011; Freese and Martin-Creuzburg, 2013; Taipale *et al.*, 2014). Bacteria have been shown to be a poor-quality food source for daphnids (Petersen *et al.*, 1978; Martin-Creuzburg *et al.*, 2011; Taipale *et al.*, 2014), although occasionally an important one during some periods (Kankaala, 1988). Nonetheless, bacteria can be useful food supplements for Daphnia; for example, the standard organic additive recommended by Baird *et al.* (1989b) and the OECD (2012) promotes bacterial growth, which is thought to aid in culture performance (Loureiro *et al.*, 2011). Reproduction and demography of *Daphnia* are closely linked to food availability (Gulati and DeMott, 1997; Kilham *et al.*, 1997; Picard and Lair, 2000; Hülsmann, 2001), and also to food quality and quantity, which could influence ecotoxicological data in assays with daphnids (Hochstädter, 2000; Antunes *et al.*, 2004).

Planctomycetes is a phylum of the domain *Bacteria* with very particular and unique characteristics, some shared in common with eukaryotes (Lage and Bondoso, 2014 and references therein). They are present in a wide range of habitats, which include aquatic and terrestrial environments, and in association with very diverse organisms (Morris *et al.*, 2006; Ward *et al.*, 2006; Webster and Bourne, 2007; Lage and Bondoso, 2011). They are normally present at low frequencies but high percentages can be found in sediments (Rusch *et al.*, 2003; Musat *et al.*, 2006; Chipman *et al.*, 2010; Hu *et al.*, 2010), in the bacterial community composition in acidic *Sphagnum* peat bogs (Dedysh *et al.*, 2006) and in the microbial community of macroalgae biofilms (Bengtsson and Ovreas, 2010; Lachnit *et al.*, 2011; Lage and Bondoso, 2011). Their ecological role is not yet well understood but the broad coverage of ecosystems suggests diverse functional niches for the planctomycetes. However, their ecological relevance in the food web as a potential food source for higher trophic levels is unknown.

Bearing in mind this lack of knowledge and the need to find alternative or complementary food sources to feed daphnids, we aimed to assess the potential of the planctomycetes as an additional or nutritive food source for *Daphnia* spp. In order to accomplish this objective, individual feeding assays, with *Rhodopirellula rubra* strain LF2 from differential growth phases (exponential and stationary), were conducted in two *Daphnia* species (*Daphnia magna* – standard species and *Daphnia longispina* – autochthonous species), and their life-history was evaluated. *R. rubra* is one of the species described from our planctomycetes collection (Bondoso *et al.*, 2014) and is being studied in various aspects (Lage *et al.*, 2013; Viana *et al.*, 2013; Flores *et al.*, 2014). Planctomycetes are easy to cultivate and allow relatively good biomass production. As cells in the exponential and stationary growth phases possess different nutritional qualities, cells from both phases were tested in this study. This is due to the production of different metabolites: in the exponential phase, the molecules produced are needed for cell growth (basic metabolism), while in the stationary phase, cells express secondary metabolism and accumulate reserves of still unknown nature in planctomycetes.

Materials and methods

Daphnia source and cultures

D. magna is a standard species used for environmental monitoring of pollutants and plays an important role in establishing regulatory criteria by government agencies (De Stasio *et al.*, 1995; Antunes *et al.*, 2007a, b; Shaw *et al.*, 2008). The experimental genotype (clone A, *sensu* Baird *et al.*, 1989a) has been cultured under laboratorial conditions for several years. *D. longispina* is a ubiquitous native species in European lentic systems, especially lakes and reservoirs; it is smaller than *D. magna* (which is a pond species), and usually more sensitive to environmental stress (Antunes *et al.*, 2007a, b). The genotype used in this study was collected in Crestuma-Lever reservoir (north of Portugal, river Douro basin) and maintained in laboratory conditions for several generations.

Monoclonal cultures of *D. magna* and *D. longispina* were reared in single-cohort group cultures under a 16^{L}:8^{D} h cycle and a temperature of $20 \pm 2\,^{\circ}C$. Rearing procedures followed those described in Antunes *et al.* (2003, 2004), Castro *et al.* (2007), and Loureiro *et al.* (2011). In brief, ASTM (1980) synthetic hard water medium was used as the culture medium, which was supplemented with a standard organic additive to provide essential microelements to daphnids. The culture medium was renewed every 2 days, and daphnids were fed with the microalgae *Raphidocelis subcapitata* (formerly known as *Selenastrum subcapitata* and *Pseudokirchneriella subcapitata*) with a ratio of 3.0×10^5 cells.mL^{-1}.day^{-1} for *D. magna*, and 1.5×10^5 cells.mL^{-1}.day^{-1} for *D. longispina*. The microalga was maintained in non-axenic batch cultures with Woods Hole MBL medium (Stein, 1973), at $20 \pm 2\,^{\circ}C$ and with a 16^{L}:8^{D} h photoperiod (~ 6000 lux). Algae were cyclically harvested while still in the exponential growth phase (5–7 days old) and inoculated in fresh medium (Environment Canada, 1992; OECD, 2006). All assays were initiated with neonates (< 24 h old), born between the 3rd and 5th broods, which were obtained from group cultures.

R. rubra cultures

R. rubra strain LF2 was grown in modified M13 culture medium (Lage and Bondoso, 2011). Cultures were kept in solid culture medium. Liquid cultures were incubated at $26\,^{\circ}C$ and 200 rpm for 3 or 7 days, respectively, for the exponential and stationary growth phases. After each growth period, cells were collected by centrifugation at 4000 r.p.m. and resuspended in distilled water and the optical density adjusted to 0.2 arbitrary units (AU) at 600 nm. The suspension was stored at $-20\,^{\circ}C$ before being used in the feeding assays.

Table 1. Experimental design of food treatments for *Daphnia* sp. assays.

	R. rubra (0.2 AU at 600 nm)			
Food treatments	25 µL of cells suspension (1/1000, v/v)	250 µL of cells suspension (10/1000, v/v)	2500 µL of cells suspension (100/1000, v/v)	*R. subcapitata*
Ctl				×
[1]	×			
[2]		×		
[3]			×	
[1]A	×			×
[2]A		×		×
[3]A			×	×

Chronic assays

To evaluate the potential of *R. rubra* as a nutritional or supplementary food source for *Daphnia* spp., two life-history assays were conducted for each species, evaluating the nutritional potential of planctomycetes in different growth phases (exponential and stationary). Feeding assays were adapted from chronic standard protocols for reproduction evaluation (ASTM, 1997; OECD, 2012). Assays were conducted for 21 days under the same temperature and photoperiod regimes described for rearing procedures. In each assay, *D. magna* and *D. longispina* were exposed to two different food scenarios (Table 1). Ten organisms born between the 3rd and 5th brood, aged < 24 h, were individually exposed to the different treatments in glass vessels filled with 25 mL of ASTM medium. As control, *Daphnia* spp. were fed with *R. subcapitata* as the only food source added. The organisms were fed daily and checked for mortality and reproductive state. In the case of neonate release, they were counted and discarded (Antunes *et al.*, 2003). The culture medium was renewed every 2 days. For each assay, the following parameters were recorded: mortality, age at first reproduction (AFR), and reproductive output. Survival and fecundity estimates were used to compute the per capita intrinsic rate of population increase (r), which was iterated from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x$$

where r is the intrinsic rate of increase (day^{-1}), x is the age class in days ($0 \ldots n$), l_x is the probability of surviving to age x, and m_x, is the fecundity at age x. Standard errors for r were estimated using the jack-knifing technique described by Meyer *et al.* (1986). For the rate of population increase, all individuals were used in the calculation because we aimed to calculate age-specific fecundity (newborns/female) and survival. These demographic parameters can only be estimated from a population ($n > 1$), so all the individuals (usually $n = 10$) from each experimental treatment contributed to the calculation. In order to test for differences in this parameter, we needed to resample the population and generate pseudo-values; in this case, we used the jack-knife method as proposed by Meyer *et al.* (1986).

The somatic growth rate was estimated from the initial and final body size of the daphnids, measured from the top of the head to the base of the caudal spine in a binocular stereoscope, according to the following expression:

$$\text{growth rate} = \frac{\ln(l_f) - \ln(l_i)}{\Delta t}$$

where l_f is the body size (mm) of the test organism at the end of the test, l_i is the average body size (mm) of a subsample ($n = 20$) of neonates coming from the same batch of neonates that initiated the test, and Δt is the time interval (in days).

Statistical analysis

All the parameters measured in the chronic assays (rate of population increase, AFR, reproductive output and somatic growth rate) were analyzed using the one-way analysis of variance test (ANOVA) to determine statistically significant differences between the food treatments. A Dunnett test (if one-way ANOVA was significant), was applied to each parameter of the two assays (exponential and stationary growth phases of *R. rubra* for each *Daphnia* species) to assess statistical differences between the different food treatments and the control.

Results

No mortality was observed in the control treatment when both species were fed with the green microalga *R. subcapitata* (Fig. 1). When fed solely with *R. rubra*, both species showed moderate to high mortality. *D. longispina* was more sensitive than *D. magna*, in both planctomycetes growth phases, as shown by overall higher mortality. Very high mortalities were observed when *D. longispina* was fed with *R. rubra* in the stationary phase. This lethal effect of *R. rubra* was, at least partially, nullified when the food ration was supplemented with *R. subcapitata*. This pattern was, however, not observed for *D. magna* fed with *R. rubra* in the exponential phase.

Figure 2 shows the results for age at first reproduction assays. In general, a significant delay of the age of reproduction was observed for *D. longispina* when fed

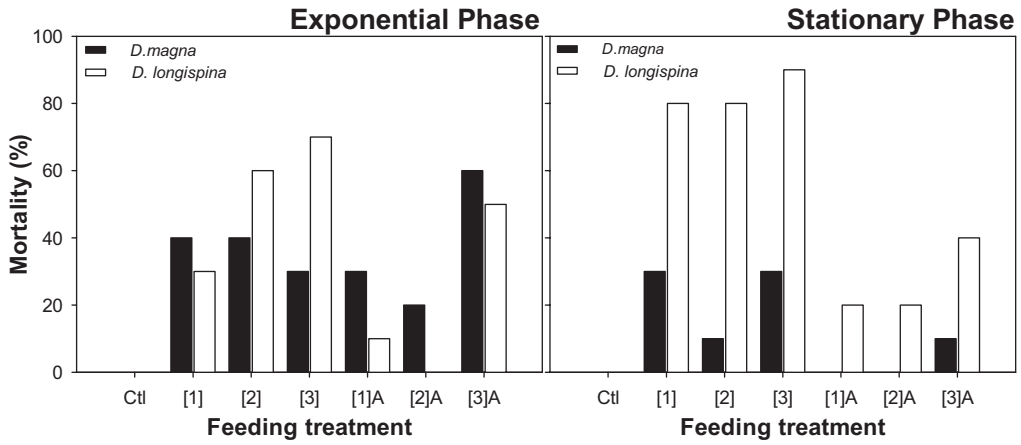

Fig. 1. Mortality levels of *D. magna* and *D. longispina* after 21 days feeding with different food sources ([1], [2], and [3] stands for 25, 250 and 2500 µL of *R. rubra* suspensions, respectively and [1]A, [2]A and [3]A stands for the same concentrations of *R. rubra* plus *R. subcapitata* ratio species).

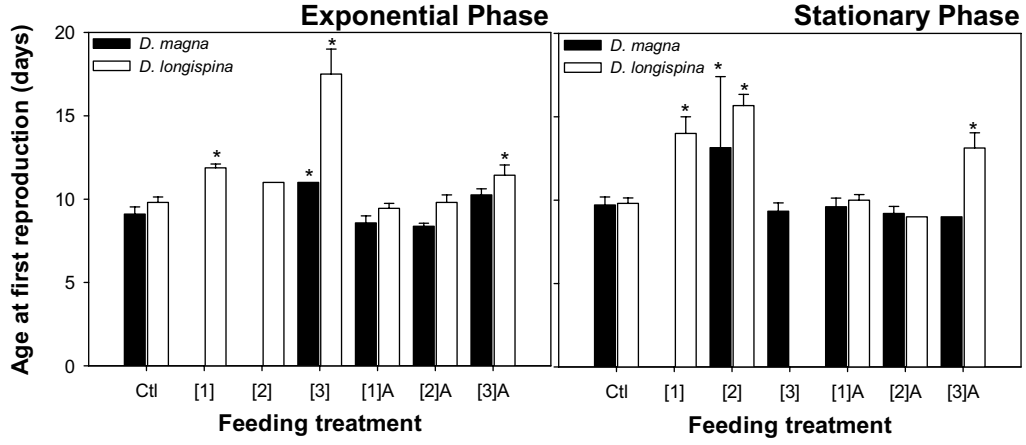

Fig. 2. Age at first reproduction parameter evaluated in *D. magna* and *D. longispina* after 21 days feeding with different food sources ([1], [2] and [3] stands for 25, 250 and 2500 µL of *R. rubra* suspensions, respectively and [1] + A, [2] + A and [3] + A stands for the same concentrations of *R. rubra* plus *P. subcapitata* ratio species). Error bars stands for standard error ($n = 10$) and *represents statistically significant differences (Dunnett test, $P \leq 0.05$) between the different feeding treatments and control.

only with *R. rubra* for both growth phases (Table 2). At the highest *R. rubra* and *R. subcapitata* concentrations, a significant delay was also observed. No reproduction or significant delay was observed for *D. magna* when fed only with *R. rubra* (Table 2). These effects were not observed when feeding was also supplemented with *R. subcapitata*.

In Figure 3 and Table 2, a life-history response of both daphnids is provided. The somatic growth rate was significantly decreased when daphnids were fed with only *R. rubra*. A similar pattern was recorded for the highest concentration of *R. rubra* + *R. subcapitata*, for both daphnid species (Figs. 3(A) and (B)). Regarding reproductive output (Figs. 3(C) and (D)), when daphnids were fed exclusively with *R. rubra*, a significant decrease was recorded. When fed with *R. rubra* and *R. subcapitata,* an increased tendency was observed for *D. magna* when *R. rubra* concentrations increased, while a significant decrease was observed for *D. longispina* for the same treatments. The analysis of the rate of population increase showed a significant decrease in daphnids fed with only *R. rubra* (Figs. 3(E) and (F)), and with *R. rubra* and *R. subcapitata* at the highest concentrations (exponential and stationary phases) only for *D. longispina*.

Discussion

Daphnid diet in culture is essentially based on a single food source (Bukovinszky *et al.*, 2012), namely the green microalgae *R. subcapitata* or *Chlorella vulgaris*. This diet limits the carbon source available for Cladocera, with possible induction of several constrains in their development and consequent population changes (Sterner *et al.*, 1993; Bukovinszky *et al.*, 2012). Several studies have already described how food limitations (in terms of quality and quantity) can induce significant alterations in *Daphnia* spp. performance, namely a delay in the growth rate, a decrease in the number of eggs produced and lower

Table 2. One-way analysis of variance (ANOVA) summary of endpoints evaluated in the life history of *D. magna* and *D. longispina* feeding with different food sources (d.f.: degrees of freedom, MS: mean square or variance, *F*: *F* statistic (MSfactor/MSresidual), *P*: probability).

	Endpoint	Species	d.f.	MS	*F*	*P*
Exponential phase	Age at first reproduction	*D. magna*	4, 31	10.628	11.569	< 0.001
		D. longispina	6, 49	22.998	17.499	< 0.001
	Somatic growth rate	*D. magna*	6, 47	0.0009	306.9	< 0.001
		D. longispina	6, 47	0.0003	31.01	< 0.001
	Total offspring	*D. magna*	6, 61	12 950	167.6	< 0.001
		D. longispina	6, 49	6757.8	60.14	< 0.001
	Rate of population increase	*D. magna*	4, 49	0.0088	3.785	0.010
		D. longispina	6, 69	0.1450	18.74	< 0.001
Stationary phase	Age at first reproduction	*D. magna*	5, 55	18.119	7.746	< 0.001
		D. longispina	5, 40	36.328	18.937	< 0.001
	Somatic growth rate	*D. magna*	6, 61	0.0006	295.5	< 0.001
		D. longispina	5, 35	0.0005	121.7	< 0.001
	Total offspring	*D. magna*	6, 65	13 053	45.73	< 0.001
		D. longispina	5, 40	5894.3	46.16	< 0.001
	Rate of population increase	*D. magna*	5, 59	0.0730	43.49	< 0.001
		D. longispina	5, 59	0.1300	17.77	< 0.001

fecundity (Lampert, 1978; Taylor, 1985; Wylie and Currie, 1991; Repka, 1997a, b; Bukovinszky *et al.*, 2012). Indeed, this fact has been a concern for researchers in the last few decades, since the health of *Daphnia* in culture may affect the results of ecotoxicological assays. This is highly relevant since *Daphnia* is the standard organism most commonly used in ecotoxicology. Thus, in order to find a viable alternative or supplementary carbon source for the diet of *D. magna* and *D. longispina*, *R. rubra* strain LF2 in the exponential and stationary growth phases were provided at different concentrations, individually and also combined with the microalgae *R. subcapitata* (the normal food source).

This study allowed assessment of the bacterial group planctomycetes both as a food supply and as a supplement to the normal food supply. On the basis of our results, in general, when daphnids were fed with only planctomycetes, the various parameters analyzed were effectively not as favorable as when they were fed with only algae. This was also demonstrated by the high mortality that occurred in all the conditions tested (Fig. 1). Planctomycetes are known to have the ability to synthesize sterols (Pearson *et al.*, 2003), which are important molecules required by daphnids (Martin-Creuzburg *et al.*, 2011) for different physiological processes, acquired only through their diet. However, our results showed that *R. rubra* could not fulfill this need even though it possesses palmitic (16:0) and oleic (18:1 ω9c) acids as the main fatty acids (Bondoso *et al.*, 2014), which are more typical of microeukaryotes than of bacteria (Kerger *et al.*, 1988). Indeed, several studies have already demonstrated the importance of sterols in the diet of *Daphnia*, since when they are exposed to poor sterol food conditions, several life-history parameters are significantly reduced (*e.g.,* somatic and population growth rates, number of eggs, viable offspring and survival) (Martin-Creuzburg *et al.*, 2005; Wacker and Martin-Creuzburg, 2007). On the other hand, the planctomycetes concentrations used in this work could potentially not have covered daphnid requirements, when added as a single food source. Several previous studies have shown that insufficient food concentration induces a significant decrease of the clutch size (Gliwicz and Guisande, 1992; Guisande and Gliwicz, 1992; Gliwicz and Boavida, 1996). Besides inadequate food concentration, some other factors could have contributed to this nutritional inability, such as inadequacy as a carbon source or toxicity induced by planctomycetes. To our knowledge, planctomycetes are not known to be noxious organisms even though studies are still lacking regarding the toxicity of these bacteria. Several studies have shown the growth impairment of *D. magna* due to toxicity induced by bacteria like *Pseudomonas* and *Hydrogenophaga,* even at low dietary concentrations (Martin-Creuzburg *et al.*, 2011; Freese and Martin-Creuzburg, 2013).

In this study, the two species of *Daphnia* showed different sensitivity to *R. rubra* as a food source. In addition to species sensitivity, a study by Repka (1997a) documented the high sensitivity of different clones of the same species (*Daphnia galeata*) to distinct food sources. In our study, *D. magna* presented better overall performance (lower mortality, no changes in life-history parameters) when fed with *R. rubra* in the stationary growth phase, while *D. longispina* preferred *R. rubra* in the exponential growth phase. When *R. rubra* was used as a supplement to *R. subcapitata*, no mortality occurred in the middle concentration of *R. rubra* in the exponential phase for *D. longispina* and in the two lowest concentrations of *R. rubra* in the stationary phase for *D. magna*. Furthermore, no significant effects on daphnid life-history were found when the rate of population increase was evaluated, with the exception of the highest concentration for *D. longispina* (Figs. 3(E) and (F)). Due to the lack of information on planctomycetes nutritional capacity in the two growth phases, we are unable to further explain the different behavior of the two daphnids. However, in the exponential growth phase, planctomycetes are essential as individual

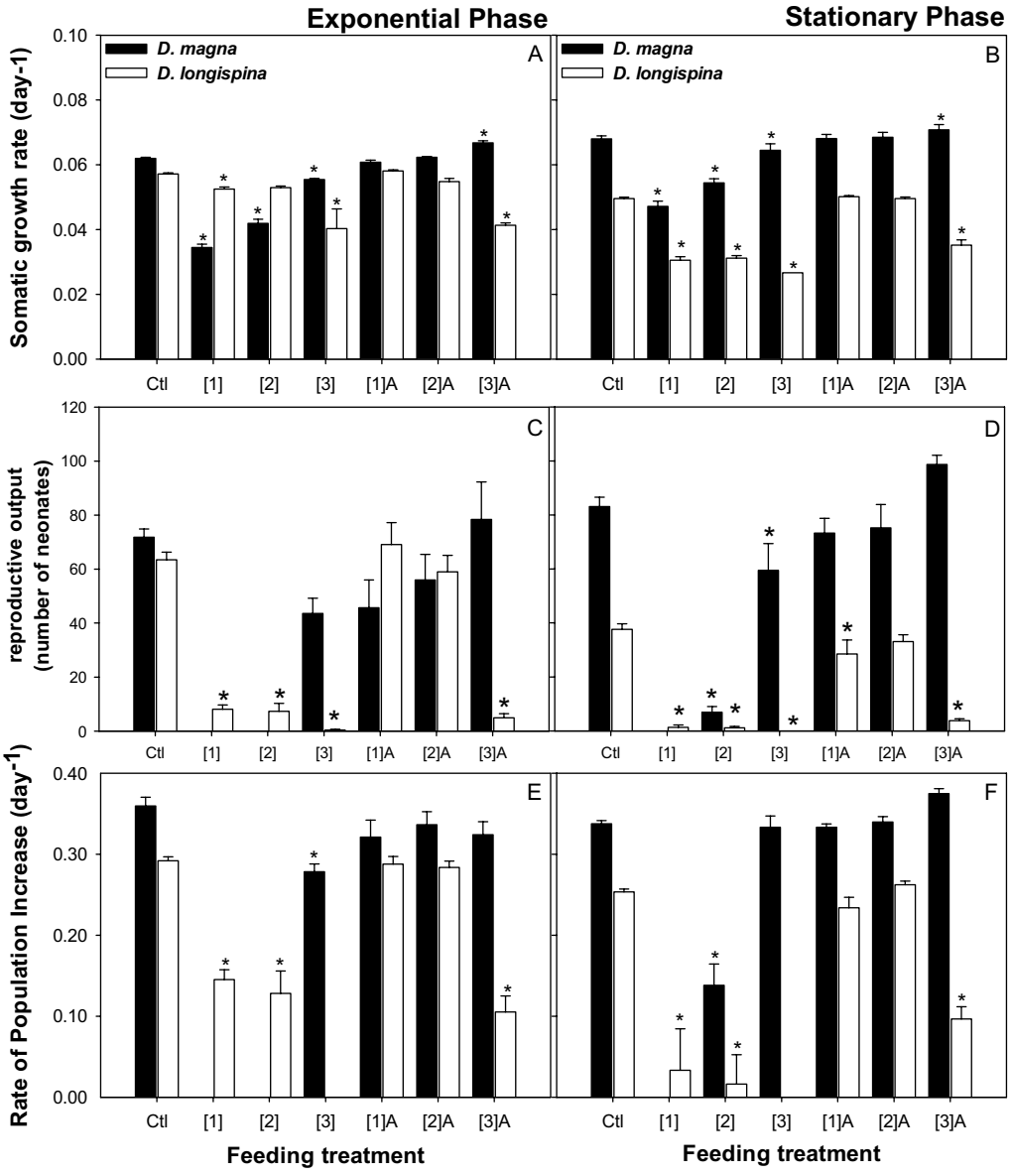

Fig. 3. Life history responses of *D. magna* and *D. longispina* after 21 days feeding with different food sources ([1], [2] and [3] stands for 25, 250 and 2500 µL of *R. rubra* suspensions, respectively and [1] + A, [2] + A and [3] + A stands for the same concentrations of *R. rubra* plus *P. subcapitata* ratio species). Error bars stands for standard ($n = 10$) error and *represents statistically significant differences (Dunnett test, $P \leq 0.05$) between the different feeding treatments and control.

unicellular forms with smaller size, which would facilitate their uptake by the smaller daphnids (*D. longispina*). A mixture of food sources seems to be nutritionally richer (high nutrient content) and increases the performance of *Daphnia* (Taylor, 1985; Sterner *et al.*, 1993; Kilham *et al.*, 1997; Bukovinszky *et al.*, 2012; Freese and Martin-Creuzburg, 2013). However, and similarly to our results, Boersma and Vijverberg (1996) observed no alterations of growth rate, reproduction and rate of population increase when *Ceriodaphnia pulchella* was fed with a mixture of two algal species instead of with a single food source. When *D. longispina* was fed with only *R. rubra,* all the parameters analysed were negatively affected. Vanni and Lampert (1992) also described a significant delay in the age at first reproduction when *Daphnia* was fed with food of low quality and quantity. Another important aspect is that this species is a much smaller Cladocera (mean adult ≈ 1.5 mm) when compared with *D. magna* (mean adult ≈ 3 mm). Being smaller, *D. longispina* may have difficulty dealing with food of large size. *R. rubra* (isolated cells lengths ≈ 1.3–2.5 µm; Bondoso *et al.*, 2014) can form aggregates of huge numbers of cells reaching sizes of more than 10 µm. Furthermore, clusters of this bacterium and the microalgae may also be formed, which may block the daphnid filtration system, with impairment of feeding function.

Another result observed was the increase in pink pigmentations in the daphnid body, especially in *Daphnia*

magna. *Daphnia*'s color depends on the food that is predominant in their diet: when daphnids feed mostly on green microalgae, they are transparent with their digestive tube colored in green or yellow, but they are white or salmon-pink when they feed on bacteria (Ebert, 2005). Well-fed animals show more vivid coloration than starved animals. In our study, we observed that *D. magna* metabolized and incorporated *R. rubra* pigments in its body since this daphnid turned overall very pinkish with the highest *R. rubra* concentrations and paler pink with the other concentrations, even though in the presence of the microalgae. This fact can be explained by the pink to reddish color of *R. rubra* (Bondoso *et al.*, 2014) and shows that daphnids have no difficulty in digesting planctomycetes.

This study showed that planctomycetes, at the concentrations tested, seem to be neither an adequate food source for daphnids, nor to improve their diet as a viable supplement. Further research is needed to increase the knowledge of these bacteria regarding toxicity and their performance as a nutritional source for other organisms, aimed at understanding their ecological role in the food web.

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