Adequacy of bacteria as supplementary food source for *Daphnia magna*

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Dissertação submetida à Faculdade de Ciências da Universidade do Porto, para a obtenção do grau de Mestre em Ecologia e Ambiente, da responsabilidade do Departamento de Biologia.

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Resumo

Daphnia é um microcrustáceo de água doce que integra o zooplâncton. Diferentes espécies são cultivadas em laboratório e utilizadas como organismos modelo em várias áreas de estudo. A alimentação deste organismo é baseada em algas, contudo, várias abordagens têm sido desenvolvidas para inferir uma dieta que melhore o seu desempenho em laboratório. Neste sentido, a qualidade nutricional da dieta fornecida às culturas de *Daphnia* pode influenciar o seu crescimento, a reprodução e a sobrevivência. Nos ecossistemas aquáticos, as bactérias fazem parte do seston e podem contribuir significativamente para a dieta do zooplâncton, nomeadamente de *Daphnia*. As bactérias apresentam elevados níveis de fósforo, suportando as altas necessidades deste composto por *Daphnia*. Além do fósforo, compostos bioquímicos como ácidos gordos e esteróis foram identificados como nutrientes essenciais na dieta deste organismo. O objetivo deste estudo foi avaliar o potencial de diferentes bactérias (a Actinobacteria *Arthrobacter* e os Planctomycetes *Gemmata obscuriglobus* e *Rhodopirellula rubra*) como fonte de alimento alternativa ou suplementar para *Daphnia magna*. Para tal, foram realizados ensaios de longa duração (21 dias) desenvolvidos de acordo com protocolos padronizados que avaliam os parâmetros da história de vida, com a alimentação por bactérias provenientes de diferentes fases de crescimento, (exponencial e estacionário). Os ensaios em que *D. magna* apenas foi alimentada com bactérias mostraram a ineficiência destas como única fonte alimentar. Contudo, quando usadas como suplemento à microalga *Raphidocelis subcapitata* (alimento padrão em cultura laboratorial) verificou-se que, para as concentrações mais elevadas, a idade à primeira reprodução diminuiu, a produção de neonatos foi significativamente mais elevada assim como o crescimento somático dos organismos e a taxa de incremento populacional. Foi ainda comprovada, visualmente, a capacidade de absorção e metabolização das bactérias por *D. magna* uma vez que a cor rosa típica das três bactérias foi incorporada pelos organismos expostos/alimentados, inclusivamente esta situação foi registada nos ovos. Os planctomycetes permitiram melhores resultados face aos da actinobacteria *Arthrobacter*, mas *G. obscuriglobus* que possui esteróis não induziu um melhor desempenho de *D. magna* comparativamente ao outro planctomycete testado (*R. rubra*). O uso da sonicação para separar as células bacterianas em agregados antes de serem fornecidas como alimento mostrou ser uma técnica eficaz. Este estudo permitiu comprovar que a adição de um suplemento alimentar bacteriano ao alimento

padrão fornecido a *D. magna* permite obter um melhor desenvolvimento e desempenho de *D. magna* em culturas laboratoriais.

Palavras-chave: *Daphnia magna*, história de vida, *Planctomycetes*, *Actinobacteria*, dieta, recursos alimentares, *Raphidocelis subcapitata*.

Abstract

Daphnia is a freshwater microcrustacean that integrates the zooplankton. Different species are cultivated in the laboratory and used as model organisms in several areas of research. The feeding based of this organism is algae, however, several approaches have been developed in order to infer a diet that improves the performance of this organism in the laboratory. In this sense, the nutritional quality of the diet provided to *Daphnia* cultures can influence growth, reproduction and survival. In natural aquatic ecosystems, bacteria are part of seston and can contribute significantly to the zooplankton diet. Bacteria present high levels of phosphorus, supporting the high needs of this compound by *Daphnia*. Beyond phosphorus, biochemical compounds as fatty acids and sterols have been identified as essential nutrients in this organism`s diet. The aim of this study was to evaluate the potential of three different bacteria (the Actinobacteria *Arthrobacter* and the Planctomycetes *Gemmata obscuriglobus* and *Rhodopirellula rubra*) as an alternative or as a supplementary food source for *Daphnia magna*. For such, long-term (21 days) assays were performed developed according to the standard protocols that evaluate the life history parameters, with the feeding by bacteria from different growth phases (exponential and stationary). The assays in which *D. magna* was only fed with bacteria showed the inefficacy of these as the only food source. However, when used as supplement to the microalgae *Raphidocelis subcapitata* (standard food in laboratory culture) it was verified that, for the highest concentrations, age at first reproduction decreased, offspring was significantly higher, and an increase of somatic growth rate and the rate of population increase. It was also visually verified the absorption and the metabolization of bacteria by *D. magna*, since the typical pink colour of the three bacteria was incorporated by the organisms, as well as by their eggs. Planctomycetes allowed better results than the Actinobacteria *Arthrobacter* sp., but *G. obscuriglobus*, that possesses sterols, did not induce a better performance compared to the other planctomycete tested (*R. rubra*). Sonication of planctomycetes food source before being provided to *D. magna* proved to be an efficient technique to separate cells in the clusters. This study confirmed that the standard food supplemented with bacteria showed a better development and performance of *D. magna* in laboratorial cultures.

Keywords: *Daphnia magna*, life-history, *Planctomycetes*, *Actinobacteria*, diet, food resources, *Raphidocellis subcapitata*.

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CHAPTER 1. Introduction

In the last century, several organisms denominated “model organisms” were used as important tools for research allowing the increase of our knowledge in numerous biological processes (Levy and Currie, 2015). Among the several model organisms, the cladoceran of the genus *Daphnia* is one of the principal standard organisms in aquatic ecotoxicology. Furthermore, this freshwater microcrustacean plays an important role in aquatic food webs as a primary consumer and is a vital food source for many higher trophic levels including fish species (Hebert, 1978; Tessier *et al*., 2000; Forró *et al*., 2008; Antunes *et al*., 2016). As main component of freshwater zooplankton (Hebert, 1978), the role of this organism is well documented in the literature and *Daphnia* is widely used in research areas such as ecology, ecotoxicology, ecophysiology, evolution, genomics, roles in host-parasite, predator-prey interactions and phenotypic plasticity (Ebert, 2005, 2011; Lampert, 2006; Stollewerk, 2010; Colbourne *et al*., 2011; Seda and Petrusek, 2011; Harris *et al*., 2012). Short life cycle, small size, high fecundity, parthenogenetic reproduction and easy maintenance in laboratory cultures are characteristics that support the significant advantages of the water flea *Daphnia magna* as a model and standard organism (Lambert, 2006; Seda and Petrusek, 2011; Antunes *et al*., 2016).

Generally, *Daphnia* species are cultivated and grown in laboratory for use as model organisms in research studies. The nutritional quality and quantity of diet provided to water fleas are important variables that not only influence their growth but also, reproduction and survival (Gulati and DeMott, 1997; Bukovinszky *et al*., 2012; Sarpe *et al*., 2014). On the other hand, the diet in *Daphnia*`s cultures may compromise its performance and the results gathered in laboratory assays (Lanno *et al*., 1989; Antunes *et al*., 2004; Ieromina *et al*., 2014). Uni-algal cultures, namely the green microalgae *Raphidocelis subcapitata* or *Chlorella vulgaris*, are currently used as the only food source for this organism under laboratory conditions (Antunes *et al*., 2004; Becker and Boersma, 2005; Choi *et al*., 2016). However, alternative or supplementary food sources should be considered in order to reduce dependence on a single food source (Bouchnak and Steinberg, 2010; Martin-Creuzburg *et al*., 2011; Buratini and Aragão, 2012; Antunes *et al*., 2016).

The search of the adequate food source for *Daphnia* culture and the way food influence the growth and reproduction of this organism has been the aim of several studies (Weers and Gulati, 1997; von Elert, 2002; Becker and Boersma, 2005; Brett *et al*., 2006; Freese and Martin-Creuzburg, 2013). Different feeding regimes are used in order to assess their contribution to the characteristics of life history of daphnids. For *Daphnia* cyanobacteria

have lower nutritional value than green algae (Arnold, 1971), and these lower nutritional value than diatoms (Choi *et al*., 2014). Infante and Abella (1985) showed that *Daphnia* size and embryo production decreased with increase density of *Oscillatoria*, which may be related to its filamentous nature and/or to chemical inhibition. The cyanobacteria, *Aphanizomenon gracile*, *Synechococcus elongatus*, and *Microcystis aeruginosa* showed to promote negative effects on survival, growth, and food uptake of *D. pulicaria*, due to size and morphological restrictions as well as nutritional inadequacy and toxicity effects (Lampert, 1981). More successful reproduction rates were obtained when *D. magna* was fed with the diatom *Stephanodiscus hantzschii* than with the green alga *Chlorella vulgaris* (Choi *et al*., 2016). These result correlated with a higher proportion of long-chain poly unsaturated fatty acids (PUFAs) in *S. hantzschii* (Choi *et al*., 2016). In addition, *D. magna* assimilated better the carbon and nitrogen originated from *S. hantzschii*. Taub and Dollar (1968) concluded that *Chlorella pyrenoidosa* and *Chlamydomonas reinhardii* are inadequate food sources for normal longevity and reproduction in *D. pulex*, reduced ovulation and an incomplete development of embryos was recorded. The performance of *D. longispina* fed with *Scendesmus quadricola*, *Oscillatoria ocutasama*, horse manure and yeast was studied by Mona *et al*. (2014). Survival, longevity and swimming activity were not affected by the different foods sources, however, the best performance of *D. longispina* was achieved with *S. quadricola* followed by the yeast, *O. ocutasama* and horse manure. Normally, the growth of *Daphnia* is determined by food availability and quality (Acharya *et al*., 2004), where phosphorus (P), and certain PUFAs are essential nutritional substances for freshwater zooplankton (Gulati and DeMott, 1997). Urabe and Sterner (2001) found that *D*. *obtusa* fed with *Scenedesmus acutus* on low N content or low P content showed slow growth, reduced eggs viability and reduced survival, mainly before maturation. The fatty acid composition of *Rhodomonas* and *Cryptomonas* are different from the *Scenedesmus* and *Chlamydomonas*, they contained high percentages of long-chained PUFAs, being considered the best food for *Daphnia* (Ahlgren *et al*., 1990).

As non-selective filter feeders, cladocerans do not discriminate between foods with regard to their nutritional quality (DeMott, 1986) and ingest small suspended organisms such as algae, bacteria, ciliates, and flagellates as well as detritus (Hebert, 1978; Hessen, 1992; Tessier *et al*., 2000). In aquatic ecosystems, particulate organic matter in suspension may contain a high proportion of bacteria, being able to maintain *Daphnia* population by itself (Picard and Lair, 2000; Freese and Martin-Creuzburg, 2013). Furthermore, when algal biomass is low or when *Daphnia* reaches a high population density, bacteria can be an important portion of the feeding of these organisms (Kankaala, 1988; Pace, 1990). As a key

species in many freshwater ecosystems, it efficiently consumes heterotrophic bacteria (Brendelberger, 1991) and this grazing is able to affect the biomass, shape the structure and species composition of the bacterial community (Jurgens *et al*., 1999; Degans *et al*., 2002). Heterotrophic bacteria are important components of aquatic food webs being responsible for the carbon and energy production and transfer in the pelagic aquatic organisms (Azam *et al*., 1983; Weisse and MacIsaac, 2000). Relatively to algae, bacteria present a higher phosphorus to carbon (P:C) ratio (Vadstein, 2000), allowing to sustain the high P *Daphnia* needs (Hessen and Andersen, 1990; DeMott *et al*., 1998). Among the various food quality parameters, besides P others elements like C and nitrogen (N) (Urabe *et al*., 1997; DeMott *et al*., 1998; Darchambeau *et al*., 2003) and organic nutrients such as PUFAs or sterols were identified as essential elements in daphnids diet (Mueller-Navarra, 1995; von Elert *et al*., 2003; Becker and Boersma, 2005; Martin-Creuzburg *et al*., 2005). PUFAs and sterols are lipids already documented to be essential in somatic growth and reproduction rates (Mueller-Navarra *et al*., 2000; von Elert *et al*., 2003). On the other hand, bacteria can be a viable source of carbon and elemental nutrients (Vadstein 2000; Biddanda *et al*., 2001), but, in general they are devoid of PUFAs and sterols (Volkman, 2003; Martin-Creuzburg *et al*., 2011). Furthermore, several studies have shown that these organisms are poor food quality for daphnids (Martin-Creuzburg *et al*., 2011; Taipale *et al*., 2012; Wenzel *et al*., 2012). Other studies already demonstrated that *Daphnia* performance is limited by the absence in sterols and PUFAs on cyanobacterial diet (Martin-Creuzburg *et al*., 2005, 2008).

Arthrobacter is usually considered a genus of soil bacteria (Hagedorn and Holt, 1975). However, representative strains have been isolated from several environments such as air, food, water, sewage, activated sludge, oil brine, plants, biofilms, cyanobacterial mats, sediment and inclusively from human clinical samples (Funke *et al*., 1996; Crocker *et al*., 2000; Irlinger *et al*., 2005; Chang *et al*., 2007; Kim *et al*., 2008; Mages *et al*., 2008; Sutthiwong *et al*., 2014). Species that are members of this genus are catalase-positive, aerobic and produce little or no acid from glucose (Chang *et al*., 2007; Sutthiwong *et al*., 2014). Phylogenetically, *Arthrobacter* sp. is *Actinobacteria* which are Gram-positive bacteria with high GC content (Eschbach *et al*., 2003). The main distinctive feature of this genus is the life cycle in which the shape of cells is typically rods during exponential growth, being replaced by cocci in the stationary phase (Sutthiwong *et al*., 2014; Busse, 2016). In natural environments they play an important role in the transformation of a wide variety of organic matter (Crocker *et al*., 2000), including aromatic hydrocarbons, that they use as the only sources of carbon or nitrogen (Stevenson, 1967; Hagedorn and Holt, 1975).

Planctomycetes is a peculiar phylum of the domain *Bacteria* characterized by members with unique morphological, genetic, metabolic and physiological identity. Their unusual complex cell plan, common budding reproduction, endocytosis capacity, eukaryote homologs of membrane-coating proteins, ability to synthesize sterols (*Gemmata obscuriglobus*; Pearson *et al*., 2003), absence of tubulin like protein FtZ (Fuerst *et al*., 2013; Sagulenko *et al*., 2014; Pinos *et al*., 2016) and crateriform pits on the cell surface (Lage *et al*., 2013), have led to a growing interest in these organisms over the last decades. Some of these features are commonly found in eukaryotes (Reynaud and Devos, 2011; Fuerst and Sagulenko, 2012). This phylum along with *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae, Poribacteria*, *OP3* and *WWE2* form the PVC superphylum (Wagner and Horn, 2006; Gupta *et al*., 2012; Lagkouvardos *et al*., 2014; Pinos *et al*., 2016). They are a ubiquitous group of bacteria, found in a great variety of ecosystems including aquatic and terrestrial ones (Winkelmann *et al*., 2010; Andrew *et al*., 2012), and in association with several organisms (Fuerst *et al*., 1997; Webster and Bourne, 2007; Lage and Bondoso, 2011; Izumi *et al*., 2013; Lage, 2013). Planctomycetes exhibit great metabolic diversity and play an important role in the global environmental cycles (Pinos *et al*., 2016 and references therein).

In laboratory, there are some variables that influence *D. magna* development. The diet provided is one of these variables and has been shown to be a determining factor in *D. magna* performance. Recent studies suggested that bacteria could be considered as supplementary and/or nutritional food for the cladocerans *D. magna* and *D. longispina* (Antunes *et al*., 2016). Although *D. magna* fed with the planctomycete *Rhodopirellila rubra* plus algae *Raphidocelis subcapitata* showed favorable growth performance on the highest concentration tested, concern was raised regarding the bacteria clusters size that could impair absorption. As a follow up, this study aims to evaluate the potential of other bacteria: the *Actinobacteria Arthrobacter*, the p*lanctomycete Rhodopirellula rubra* strain LF2 and the sterol producing p*lanctomycete Gemmata obscuriglobus* as an additional and/or nutritional food sources for *D. magna*. Furthermore, the effect of sonication to separate bacteria in clusters before feeding *D. magna* was also tested. For such, long-term (21 days) assays will be performed, developed according to standard protocols, and evaluated the life history parameters after feeding from different bacterial growth phases (exponential and stationary).

CHAPTER 2. Material and Methods

2.1 *Daphnia magna* cultures

Cultures of *D. magna* (clone A, *sensu* Baird *et al*., 1989a), was grown under controlled laboratory conditions for several generations as pure parthenogenic cultures. Monoclonal cultures of *D. magna* were reared in laboratory at temperature (20±2 ºC), photoperiod ($16h^{L}$:$8h^{D}$) and maintained in ASTM synthetic hard water medium (ASTM, 1980). *D. magna* cultures were supplemented with a standard organic additive (suspension extracted from the brown algae *Ascophyllum nodosum*) (Baird *et al*., 1989b), which provides essential micronutrients to *Daphnia*. The cultures were fed with the microalgae *Raphidocelis subcapitata* (formerly known as *Selenastrum subcapitata* and *Pseudokirchneriella subcapitata*) with a ratio of 3.0 x 10^5 cells.mL^{-1}.day^{-1}. The microalgae was kept in cultures in Woods Hole MBL medium (Stein, 1973), under controlled conditions of temperature (20±2 ºC) and photoperiod $16h^{L}$:$8h^{D}$ (~ 6000 lux), being harvested in the exponential growth phase (5 – 7 days) (Environment Canada, 1992; OECD, 2006). Algal cell concentrations were calculated based on the correlation of absorbance measured at λ=440 nm and cell density previously determined. *Daphnia magna* cultures were fed three times a week when the culture medium was renewed. Neonates born between the 3rd to 5th broods were used for renewing the culture or for initiating assays.

2.2 *Arthrobacter* sp., *Gemmata obscuriglobus* and *Rhodopirellula rubra* cultures

The procedures for culturing the three bacteria were similar. Specific media were used to grown the different bacteria: *G. obscuriglobus* was cultured in nutrient agar (NA) and nutrient broth (NB), *Arthrobacter* sp. was grown in M14 medium (Lage and Bondoso, 2011), and *R. rubra* was cultured in the medium M13 (Lage and Bondoso, 2011). All bacteria were grown at 26 ºC, first in solid culture medium and then upscaled in liquid media to a volume of 600 mL in a continuous stirring of 200 rpm. After 3 or 7 days of growth for exponential and stationary phases, respectively, the cells were collected by centrifugation at 4000 rpm. Cell pellets were resuspended in distilled water and the optical density at λ=600 nm adjusted to 0.2 arbitrary units (AU). The all cell suspensions were stored at -20 ºC for posterior use in the feeding assays (*see* below chronic assays). Due to the clustering of the cells, 50 mL planctomycetes

cell suspensions were defrost and sonicate for 30 s, in a Misonix Microson Ultrasonic Cell Disruptor XL set to intensity 10 watts. Next, the cell suspensions were adjusted to 0.2 arbitrary units (AU) at λ=600 nm before provided as food to the *D. magna* assays.

2.3 Chronic assays

To evaluate the potential of *Arthrobacter* sp., *G. obscuriglobus* and *R. rubra* to be used as additional or as nutritional food source for *D. magna* in laboratory cultures, two distinct conditions of each bacteria were tested: 1) bacteria from exponential growth phase; and 2) bacteria from stationary growth phase. Feeding assays were adapted from chronic standard protocols for life-history parameters evaluation (ASTM, 1997; OECD, 2012). *D. magna* in the feeding assays were kept under the same temperature, photoperiod regimes and medium renewed with a standard organic additive as described for rearing cultures. In each assay, 7 food conditions were tested: 1) a negative control fed with *R. subcapitata* with a ratio of 3.0×10^5 cells mL^{-1} day^{-1}; 2) three different concentrations of bacteria (25, 250 and 2500 µL of cells suspension stands for 1/1000, 10/1000 100/1000, v/v), herein designated [1], [2] and [3], and 3) the same bacteria concentrations described above (25, 250 and 2500 µL of cells suspension) adding a standard food ratio of *R. subcapitata* (3.0×10^5 cells mL^{-1} day^{-1}), herein designated by [1]+A, [2]+A, and [3]+A.

Ten individualized *D. magna* replicates were used in each treatment. All assays started with neonates born between the 3^{th} and 5^{th} brood, and an aged with less than 24 h. Assays were conducted for 21 days in glass vessels in a final volume of 50 mL of ASTM synthetic hard water medium. Organisms were fed with alga every day (bacteria were given only when the culture medium was changed, every two days), the medium was renewed every two days, and the assays were monitored daily for mortality and reproductive state. Neonates born during the assay were counted and discarded. For each assay, several endpoints were quantified: mortality (%), age at first reproduction (days), somatic growth rate (day^{-1}), reproductive females (%), reproductive output and rate of population increase (r, day^{-1}).

For somatic growth rate calculation, *D. magna* body length was measured, from the top of the head to the base of the caudal spine (Fig. 1). An average of initial body length was measured at the beginning of the assay in a sub-sample of 20 neonates from the same brooding of the organism used in the assay. At the end of the test (21 days) all the survivor organisms were measured in a binocular stereoscope. The somatic growth rate was calculated, according to the following expression:

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

where l_f is the body length (mm) of the organism after 21 days of the assay, l_i is the average body length (mm) of a subsample (n=20) of neonates coming from the same batch of neonates that initiated the test, and Δt is the duration of the test (in days).

Survival and fecundity related data were used to compute for the estimation the per capita intrinsic rate of population increase (r), which was iterated from the Euler–Lotka equation:

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

where r is the intrinsic rate of population increase (day^{-1}), x is the age class (in days), l_x is the probability of surviving at 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).

Fig. 1 – *Daphnia magna*. Body length is measured from the top of the head to the base of tail spine.

2.4 Statistical analysis

One-way Analysis of Variance (ANOVA) was used to analyze the endpoints measured in the long-term (21 days) assays (age at 1st reproduction, somatic growth rate, reproductive output

and rate of population increase). This analysis allows to determine statistically differences between the food treatments. If ANOVA was significant a Dunnett test was conducted to assess statistical differences between the different food treatments and the control. For all analyses, the level of significance (α) used was 0.05.

CHAPTER 3. Results

By visual assessment, *D. magna* fed with *R. subcapitata* (control), has no additional body coloration besides green/brown color in the gut due to the alga and the organic additive. However, when *D. magna* was fed with any of the bacteria it exhibits a pink coloration due to bacterial pigments that increased with bacterial concentrations. This pink coloration was even clearly evident in the eggs in the marsupial camera (Fig. 2).

Table 1 shows the results of mortality, age at first reproduction and percentage of reproductive female in the different assays. When compared to Planctomycetes, higher levels of mortality were observed when *D. magna* was fed with *Arthrobacter* sp. in both growth phase assays. Higher mortality was also observed in *G. obscuriglobus* in the highest concentration tested for both growth phases when *D. magna* was fed with only bacteria. However, when this bacterial concentration was added with alga no mortality occurred.

Table 1 – Results of 21 days feeding assays of *Daphnia magna* fed by *Arthrobacter* sp., *Gemmata obscuriglobus* and *Rhodopirellula rubra* strain LF2 in Exponential and Stationary growth phase: Mortality (%), Age at 1st reproduction (days) and Reproductive females (%). ‡ stands for no reproduction recorded. † only one female reproduced. *represents statistically significant differences (Dunnett test, $P \leq 0.05$) between the different feeding treatments and control.

		Exponential phase			Stationary phase		
	Treatments	Mortality	Age at 1st reproduction	Reproductive females	Mortality	Age at 1st reproduction	Reproductive females
G. obscuriglobus	Ctl	0	10.1±0.10	100	0	10.2±0.13	100
	[1]	0	>21days‡	0	0	>16days	10
	[2]	10	14.1±0.35	100	0	12.4±0.34	100
	[3]	70	12.3±0.25	40	100	13days†	10
	[1]+A	0	9.7±0.15	100	0	10.0±0.00	100
	[2]+A	0	9.6±0.16*	100	10	9.9±0.11	90
	[3]+A	10	9.0±0.00*	100	0	9.8±0.20	100
R. rubra	Ctl	0	10.9±0.10	100	0	11.1±0.31	100
	[1]	0	≥ 21days	10	0	>21days‡	0
	[2]	0	13.8±0.66	100	10	13.6±0.48	90
	[3]	0	13.7±0.42	100	10	13.0±0.47	90
	[1]+A	0	10.8±0.25	100	0	10.8±0.20	100
	[2]+A	0	9.8±0.25*	100	10	10.1±0.31*	90
	[3]+A	0	10.0±0.26*	100	0	9.6±0.22*	100
Arthrobacter	Ctl	10	10.8±0.13	100	10	10.6±0.16	100
	[1]	30	>21days‡	0	20	≥21 days	30
	[2]	30	>21days‡	0	0	13.2±0.65	60
	[3]	100	---	0	20	>21days‡	0
	[1]+A	70	10.8±0.13	100	30	11.0±0.00	100
	[2]+A	30	11.0±0.41	90	40	9.9±0.11	70
	[3]+A	40	10.5±0.38	80	10	9.8±0.20*	100

In the assays where *D. magna* was fed with only bacteria, a significant delay in the age at first reproduction was observed for all bacteria. When *D. magna* was fed with Planctomycetes as supplementary to the standard food source, the age at first reproduction occurred significantly earlier, *G. obscuriglobus* (exponential phase: $F_{(3,\ 36)} = 13.78$, $P < 0.001$), *R. rubra* (exponential phase: $F_{(3,\ 36)} = 6.149$, $P = 0.002$; stationary phase: $F_{(3,\ 35)} =$

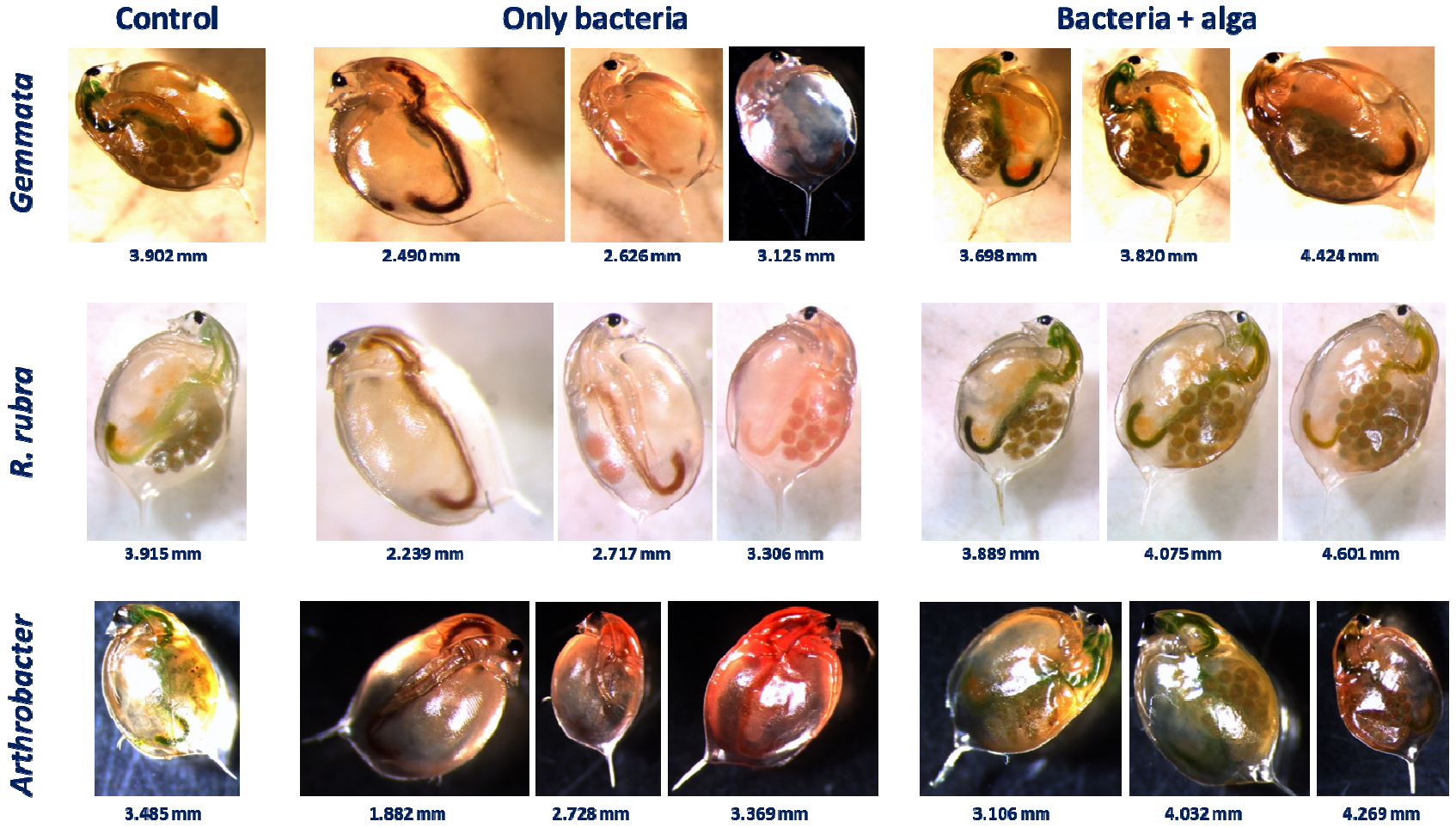

Fig. 2 - *Daphnia magna* female after 21 days fed with different food treatments evidencing the pink or green coloration due to the bacteria or alga respective food sources. Under each image, the body length (mm) of the organism is presented. Control: *D. magna* fed with *R. subcapitata*; Only bacteria: *D. magna* fed with *G. obscuriglobus*, *R. rubra* and *Arthrobacter* sp. with different concentrations 25 µL, 250 µL and 2500 µL; Bacteria + alga: *D. magna* fed with *G. obscuriglobus*, *R. rubra* and *Arthrobacter* sp. with different concentrations 25 µL, 250 µL and 2500 µL + *R. Subcapitata*.

6.693, $P < 0.001$). This was also observed for *Arthrobacter* sp. in stationary growth phase ($F_{(3, 33)} = 8.921$, $P < 0.001$).

No effects were observed in the number of reproductive females when *D. magna* food source was supplemented with Planctomycetes in both growth phases (Table 1). When Planctomycetes were used as the only food source, *D. magna* showed a decreased reproductive capacity, which was more evident in the lowest concentration tested. Even though high *Arthrobacter* sp. mortality was observed in some treatments where this bacterium was added as a supplement, reproduction occurred while females were still alive. In general, when *D. magna* was only fed with *Arthrobacter* sp. no reproduction was observed namely in exponential growth phase.

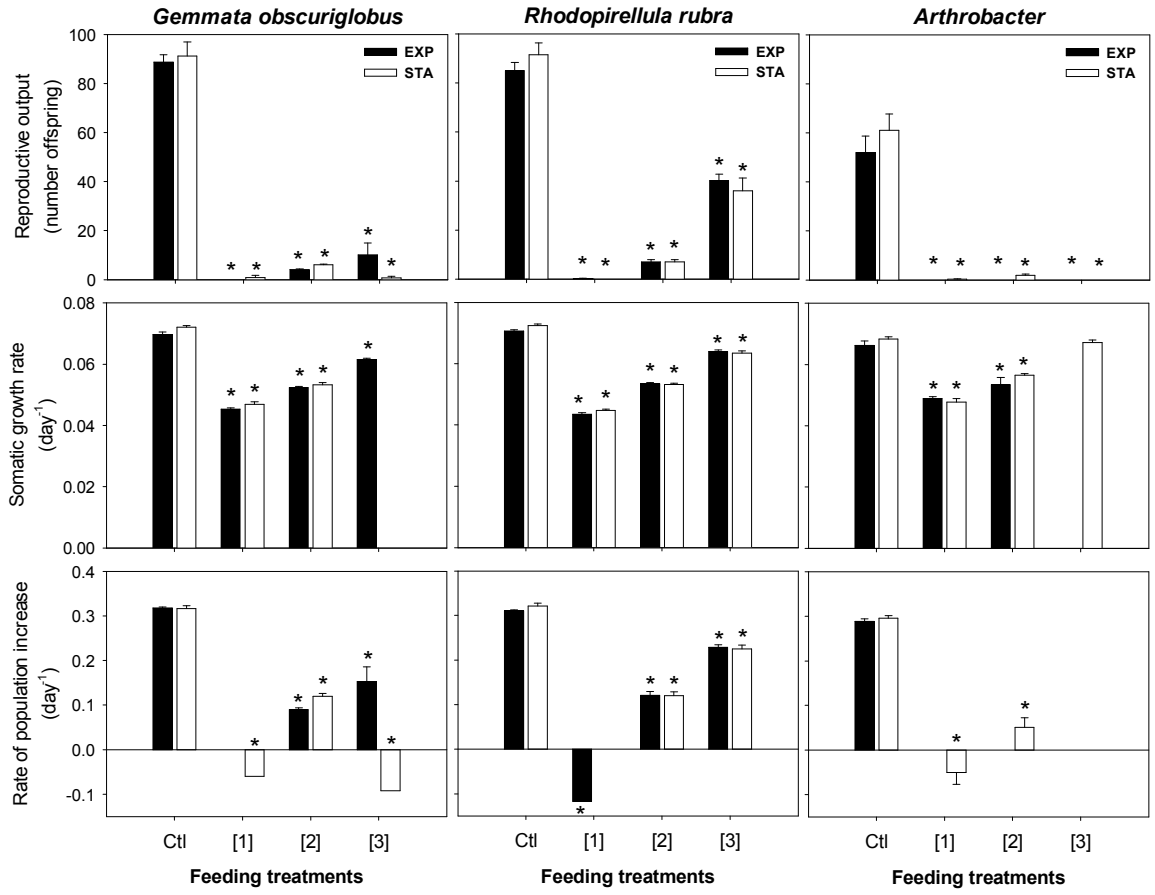

Fig. 3 - Life history responses of *Daphnia magna* after 21 days feeding with different food sources ([1], [2] and [3] stands for 25, 250 and 2500 µL, respectively of *G. obscuriglobus, R. rubra* and *Arthrobacter* sp. suspensions). Error bars stands for standard error ($n = 10$) and *represents significant differences (Dunnett test, $P \leq 0.05$) between the feeding treatments and control group.

Figure 3 and Table 2 show the results of reproductive output, somatic growth rate and the rate of population increase when *D. magna* was fed with only bacteria. A significant decrease of all parameters measured was observed in both growth phases for all

concentration tested. The less negatively impacted treatment was observed in the highest *R. rubra* concentration for both growth phases in all the parameters analyzed.

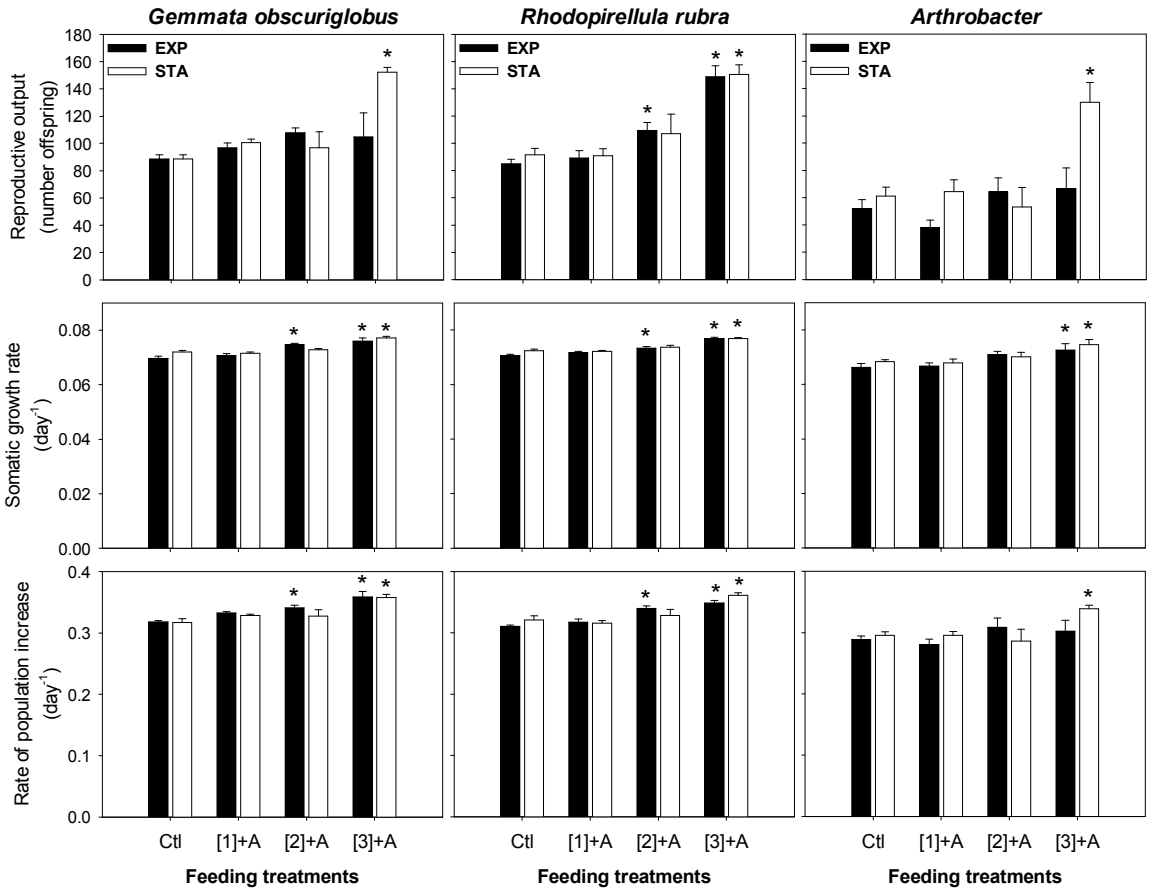

Fig. 4 - Life history responses of *Daphnia magna* after 21 days feeding with different food sources ([1]+A, [2]+A and [3]+A stands for 25, 250 and 2500 µL respectively of *G. obscuriglobus, R. rubra* and *Arthrobacter* sp. suspensions plus *R. subcapitata* ratio). Error bars stands for standard error (n = 10) and *represents significant differences (Dunnett test, $P \leq 0.05$) between the feeding treatments and control group.

Figure 4 and Table 2 show the same parameters of Fig. 3, in this case the treatments when bacteria were used as supplementary food source. In general, for all bacteria the parameters measured showed a slight increase along the three concentrations tested. All parameters were significantly increased when *D. magna* was fed with the highest bacterial concentration from stationary growth phase. This is also true for *R. rubra* in the two highest concentrations for exponential growth phase. Also, a significant increase was observed for somatic growth rate and rate of population increase in *G. obscuriglobus* at the middle and highest concentrations for exponential growth phase. Furthermore, *Arthrobacter* sp. induced a significant increase in somatic growth rate at exponential growth phase in the highest concentration tested.

Table 2 - One-way analysis of variance (ANOVA) summary of endpoints evaluated in the life history of *Daphnia magna* feeding with different food sources (d.f.: degrees of freedom, *F*: F statistic (MSfactor/MSresidual), *P*: probability) NS – non-significant.

		Endpoint	Bacteria	d.f.	*F*	*P*
Exponential phase	**algae**	Reproductive output	*Arthrobacter* sp.	3, 36	60.57	< 0.001
			G. obscuriglobus	3, 36	218.4	< 0.001
			R. rubra	3, 36	310.9	< 0.001
		Somatic growth rate	*Arthrobacter* sp.	2, 18	28.28	< 0.001
			G. obscuriglobus	3, 28	423.7	< 0.001
			R. rubra	3, 36	641.5	< 0.001
		Rate of population increase	*Arthrobacter* sp.			
			G. obscuriglobus	2, 27	36.55	< 0.001
			R. rubra	3, 35	1216	< 0.001
Stationary phase		Reproductive output	*Arthrobacter* sp.	3, 36	83.60	< 0.001
			G. obscuriglobus	3, 36	223.8	< 0.001
			R. rubra	3, 36	138.8	< 0.001
		Somatic growth rate	*Arthrobacter* sp.	3, 30	136.1	< 0.001
			G. obscuriglobus	2, 27	360.6	< 0.001
			R. rubra	3, 34	489.0	< 0.001
		Rate of population increase	*Arthrobacter* sp.	2, 27	79.23	< 0.001
			G. obscuriglobus	3, 34	1544	< 0.001
			R. rubra	2, 27	157.0	< 0.001
Exponential phase	**bacteria + algae**	Reproductive output	*Arthrobacter* sp.	3, 36	1.811	NS
			G. obscuriglobus	3, 36	0.867	NS
			R. rubra	3, 36	24.04	< 0.001
		Somatic growth rate	*Arthrobacter* sp.	3, 19	3.420	NS
			G. obscuriglobus	3, 35	15.90	< 0.001
			R. rubra	3, 36	32.14	< 0.001
		Rate of population increase	*Arthrobacter* sp.	3, 36	0.979	NS
			G. obscuriglobus	3, 36	11.17	< 0.001
			R. rubra	3, 36	22.01	< 0.001
Stationary phase		Reproductive output	*Arthrobacter* sp.	3, 36	9.044	< 0.001
			G. obscuriglobus	3, 36	16.51	< 0.001
			R. rubra	3, 36	10.40	< 0.001
		Somatic growth rate	*Arthrobacter* sp.	3, 26	4.794	= 0.009
			G. obscuriglobus	3, 35	30.01	< 0.001
			R. rubra	3, 35	17.89	< 0.001
		Rate of population increase	*Arthrobacter* sp.	3, 36	4.781	= 0.007
			G. obscuriglobus	3, 36	6.713	< 0.001
			R. rubra	3, 36	9.690	< 0.001

CHAPTER 4. Discussion

Daphnia magna has been used as a model organism recommended for standardized procedures for ecological risk assessments of surface waters in many countries (Jonczyk and Gilron, 2005, OECD, 2012). The success of *Daphnia* cultures in the laboratory depends on culture conditions, mainly water quality and diet (Elendt and Bias, 1990). Diet must take into account the nutritional needs of the species, in order to improve its performance and not induce alterations that could affect research. Under laboratorial conditions, *Daphnia* diet is based on a single food source as previously referred. In order to find an additional and/or nutritional food source for this organism in our work different bacterial species in exponential and stationary growth phases, at different concentrations were supplied individually and in supplement to the standard food source, the microalgae *R. subcapitata.* The here-obtained results show that, the life-history of *D. magna* was influenced by the quantity and the quality of the available food sources.

When compared to control (*R. subcapitata* as only food source), all bacteria were not able to provide an adequate quantitative and/or qualitative food supply for *D. magna*. All the parameters showed reduced values comparative to the control (Figure 3, Table 1). Several studies have described heterotrophic bacteria as being of poor food quality for daphnids (Martin-Creuzburg *et al*., 2011; Wenzel *et al*., 2012; Taipale *et al*., 2012; Antunes *et al*., 2016). The lack of sterols and the deficiency of PUFAs in bacteria are mentioned by several authors as the major food quality constraints (von Elert *et al*., 2003; Martin-Creuzburg *et al*., 2008, 2011; Freese and Martin-Creuzburg, 2013). A diet deficient in these essential elements has been shown to affect the performance of daphnids (Martin-Creuzburg *et al*., 2009). When *D. magna* was fed exclusively with *G. obscuriglobus*, the best performance was obtained at the middle concentration tested in both phases, once low or no mortality was observed and all females reproduced. Moreover, higher values of mortality were observed at the highest concentration in both phases, a result that is beyond our comprehension. When *R. rubra* was provided as the only food source, the best performance was obtained at the two highest concentrations tested in both phases, where mortality was negligible and, in general, all females reproduced. The somatic growth rate in both Planctomycetes was reduced, but with the increase of food concentration a slight improvement was observed. Regarding food quality, *G. obscuriglobus*, contrary to *R. rubra*, has the ability to synthesize sterols (lanosterol and parkeol; Pearson *et al*., 2003), which is a typical characteristic of eukaryotes and unusual among bacteria (Fuerst and Sagulenko,

2011). However, our results showed that *R. rubra* induced a better performance in the growth, survival and production of neonates of *D. magna,* and *G. obscuriglobus* seem not to satisfy the physiological requirements of *D. magna*. *R. rubra* possesses palmitic (16:0) and oleic (18:1 ω9c) acids as its main fatty acids (Bondoso *et al*., 2014). Furthermore, our results showed a better performance of *D. magna* when fed exclusively with Planctomycetes than the ones obtained in the study performed by Wenzel *et al*. (2012). When only *Pseudomonas* was supplied to *D. galeata*, they reported the death of all daphnids when and reproduction was only observed on a diet containing at least 50% *Rhodomonas* (Wenzel *et al*., 2012). In contrast, Bednarska *et al*. (2014) showed that *Daphnia* could grow and reproduce when fed solely on the cyanobacteria *Cylindrospermopsis raciborskii* (a non-toxic strain). Similarly, when *D. magna* fed exclusively on Planctomycetes, our results showed an increase in the age at first reproduction, a decrease in the number of neonates and a reduction in somatic growth rate. Comparatively to *Arthrobacter* sp., *G. obscuriglobus* and *R. rubra* sustained better the survival, growth and reproduction of *D. magna* when used as the only source of food. Inadequacy in the concentrations tested or other confounding factors, not measured in our experiment, could have contributed to the low performance of *D. magna* when fed on only the three bacteria tested. Alternatively, other bacterial concentrations or the supply of bacteria every day should be tested in order to verify if the performance of *D. magna* life-history could be improved.

However, this study showed that bacteria are good food sources in supplement to the standard food provided in laboratorial cultures for *D. magna*. Depending on the bacterium and its concentration, *D. magna* performance was significantly improved as compared to the control (green algae, *R. subcapitata* used as the only food source). For all bacteria the highest concentration tested or even the middle concentration, the reproductive output, the somatic growth rate and the rate of population increase were significantly improved especially when Planctomycetes were used. Our results are consistent with previous studies showing that, when in supplement, bacterial diet can improve the performance of *Daphnia* (Taipale *et al*., 2012; Wenzel *et al*., 2012; Antunes *et al*., 2016). For Wenzel *et al*. (2012) the life-history characteristics of daphnids depend on the association between heterotrophic bacteria and phytoplankton. Freese and Martin-Creuzburg (2013) also described an increase in *Daphnia*`s somatic growth rates when heterotrophic bacteria were added in food as compared with a single algae diet. For the authors, bacteria can provide essential nutrients not available in algae. Different responses were observed when *D. magna* was fed with the different bacteria tested. *D. magna* showed greater sensitivity to *Arthrobacter* sp. (highest mortality) than to the Planctomycetes. Taipale *et al*. (2012)

documented that the dietary quality of different types of bacteria varies in their ability to support *D. magna* survival, growth and reproduction. The literature is scarce regarding the nutritional values of *Actinobacteria* and Planctomycetes as food source for *Daphnia*. In our work, *D. magna* presented the best responses of the life-history parameters when fed with Planctomycetes in the exponential growth phase, while *Arthrobacter* sp. present best results in the stationary growth phase. This result is not in agreement with the previous results by Antunes *et al*. (2016), where *D. magna* performance was better improved with *R. rubra* in the stationary growth phase. These contradictory results need confirmation. The lack of information in the literature on the nutritional capacity of bacteria (*Arthrobacter* sp. and Planctomycetes) in the two growth phases does not allow us to explain the preference of *D. magna*.

Different zooplankton species have different ability to ingest bacterial cells (Bouvy *et al*., 1994). Filamentous or aggregated forms may mechanically interfere with the filtration process, reducing food intake (Bednarska et al., 2014 and references therein). The Planctomycetes can form rosette-like aggregates with large numbers of cells (> 50 cells) as already reported by Bondoso *et al*. (2014). In order to reduce this aggregation, a sonication process was used. This methodology was introduced because in a previous study (Antunes *et al*., 2016) a hypothesis was raised about the incapability of *Daphnia* to feed on the planctomycetes aggregate. Our results where *Daphnia* was fed with bacteria previously treated with sonication showed that this technique improved the performance of *D. magna*.

Generally, algae are considered as the fundamental food source for daphnids, while heterotrophic bacteria are considered less important (Martin-Creuzburg *et al*., 2011). Besides phytoplankton, bacteria may be an alternative food source for zooplankton (Onandia *et al*., 2015). Evidence was already demonstrated in numerous studies in which *Daphnia* was able to consume bacteria (Jurgens *et al*., 1994; Langenheder and Jurgens, 2001; Degans *et al*., 2002; Pernthaler *et al*., 2004). Pedrós-Alió and Brock (1983) reported an increase of the normal growth and reproduction of *D. longispina* when it was fed with only bacteria. According to Picard and Lair (2000), *Daphnia* has the capacity to grow and reproduce in low concentrations of bacteria, being an important aspect for its suitability under competitive conditions. These authors also reported that bacterial P plays an important role in the growth of *Daphnia*. In addition, bacterial carbon also may be transferred directly to macrozooplankton by daphnids (Work and Havens, 2003 and references therein). The contribution of heterotrophic bacteria to the nutrition of *Daphnia* species in aquatic ecosystems is supported by analysis of stable isotope patterns and fatty acids biomarkers (Perga *et al*., 2006; Taipale *et al*., 2008, 2009).

Another factor to be accounted in daphnids diet is the food level. For Gliwicz and Lampert (1990), growth and reproduction in zooplankton depend on the minimum food concentration. *D. pulicaria* under decreased food concentration showed reduction in body length, protein content, lipid content, body carbon, dry mass, clutch size and an increase in age at first reproduction (Guisande and Gliwicz, 1992). Our results are in agreement with these studies where an increase of growth and reproduction in *D. magna* was observed in both growth phases with increasing Planctomycetes concentrations. Furthermore, the number of reproductive females remained the same at the two highest concentrations in both phases when only *R. rubra* was provided.

Daphnia acquires the colour of the food ingested as already described in other studies (Ebert, 2005; Antunes *et al*., 2016). In our study, we observed that *D. magna* metabolized and incorporated the pink pigments of the bacteria into its body and eggs. Increasing intensity of the pink color was observed with increasing bacterial concentrations, even though in the presence of the microalgae. This result shows that *D. magna* has no difficulty to ingesting *Arthrobacter* sp. and Planctomycetes.

CHAPTER 5. Conclusions

According to our results, we conclude that daphnids are able to feed on bacteria as the only food source although, in the concentrations tested, the life-history responses of *D. magna* were deficient. Bacteria supplemented to algae (standard food source *R. subcapitata*) showed to be an adequate diet for daphnids with a significant improve of life-history parameters, namely in the two highest bacterial concentration tested. Planctomycetes were more efficient in improve *D. magna* performance than the Actinobacteria *Arthrobacter* sp.. Particle food size, namely Planctomycetal aggregates, affects the life-history parameters of *D. magna*, as proved by the sonication of food supply. This technique minimized the aggregate forms promoting a better ingestion of the bacteria by *D. magna*

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