Contribution of bacteria to *Artemia* nutrition in conditions of degressive algal feeding

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Abstract: *Artemia* were reared, under zero water exchange, on four different microalgae feeding regimes: standard ad libitum feeding (SF1), half of SF1 (SF1/2), one third of SF1 (SF1/3), and a quarter of SF1 (SF1/4). In additional treatments, for each of these feeding regimes nitrogen waste from *Artemia* in the cultures was converted into bacterial biomass by addition of carbohydrate to produce carbon/nitrogen (C/N) ratio 10. The objective was to investigate what the contribution of bacteria to the *Artemia* diet was at different degressive algae feeding rations. After 15 days of culture period, the results of survival and biomass production (WW g/L) indicated that stimulating bacterial growth increased survival and biomass production of *Artemia* in all feeding rations when compared to the corresponding control treatments with algae only. Particularly, manipulating the C/N ratio in the SF1/3 treatment induced the improvement of *Artemia* biomass production equal to that obtained in the treatment where *Artemia* were offered solely algae at SF1 ration. Moreover, the results of fatty acid assimilation and especially 15N accumulation in *Artemia* indicated that *Artemia* utilized relatively more bacteria for the treatments with the lowest algae supply. These findings open perspectives for using bacteria to substitute or supplement microalgae in *Artemia* biomass production.

Index term: *Artemia*, biomass production, C/N ratio,

I. INTRODUCTION

Among live food organisms, *Artemia* nauplii are extensively used for the larval stages of fish and crustaceans, because they satisfy the nutritional requirements, are convenient to use and are readily available as dry cysts (Sorgeloos *et al*., 2001). Moreover, the protein content of on-grown *Artemia* is around 50% (Anh *et al*., 2009a); therefore it can be a candidate to replace fish meal in aquafeed ingredients, and adult *Artemia* is a good food to enhance maturation of marine shrimp (Gelabert *et al*., 2003).

Artemia can be cultured with selected algae (Fábregas *et al*., 1996; Thinh *et al*., 1999), but it is costly to offer *Artemia* only microalgae under tank culture systems (Lavens and Sorgeloos, 1991). Thanks to its particular biological characteristics, it can also be cultured with low nutrient diets from cheap agriculture by-products, which reduces the production costs, e.g. rice bran, soybean meal (Anh *et al*., 2009b), animal wastes (Baert *et al*., 1997), aquaculture wastes (Marinho-Soriano *et al*., 2010), yeasts (Coutteau *et al*., 1990), and inert diets (such as wheat flour, fishmeal, egg-yolk, homogenized liver and rice powder) (Dobbeleir *et al*., 1980).

As is the case with *Artemia* culture, foods are also the major component of aquaculture production costs in general, with food conversion efficiency of aquatic animals in the range 1-3 (Naylor *et al*., 2000). The unutilized nitrogen and phosphorus of food remain as waste in the water (Piedrahita, 2003).

Along with the present drive to commercialized aquaculture, research efforts have focused to increase production per culture volume, to reduce production costs and to reduce nitrogen discharge from aquaculture to the environment. This can be accomplished by, amongst others, retaining more nitrogen from the food in the culture animal via the stimulation of the production of natural protein-rich food sources using the heterotrophic bacteria/biofloc technique. Bacteria, which range in size between 0.6-3 µm, can be used as food for *Artemia* (Yasuda and Taga, 1980; Douillet, 1987; Intriago and Jones, 1993; Gorospe *et al*., 1996). To enhance bacterial growth, it is recommended to maintain the carbon/nitrogen (C/N) ratio at 10 in the culture system (Avnimelech, 1999). Therefore, carbohydrate is added to the system (Burford *et al*., 2004; Avnimelech, 2007; Crab *et al*., 2007; Schneider *et al*., 2007; Nootong *et al*., 2011). Bacteria aggregate with other particles such as colloids, organic polymers, and microalgae and diverse mixtures of bacteria produce flocs (De Schryver *et al*., 2008), with a size generally between 0.1 to a few mm (Avnimelech, 2011). The consumption of bioflocs by fish or shrimp has demonstrated many benefits such as significantly reduced production costs and improved water quality (Avnimelech, 1999; Burford *et al*., 2004; Hari *et al*., 2004, 2006; Crab *et al*., 2009; Nootong *et al*., 2011).

In previous study (Toi *et al*., 2013) the result demonstrated that bacteria grown while manipulating the C/N ratio at 10, partially compensated for nutrition of *Artemia* under limited algae supply. However, this study applied a low algal feeding regime which was fixed at random at ¼ of a reference regime, and didn't point out from which level of reduction of algal supply onwards *Artemia* takes advantage of the bacteria supply. Therefore, in the present study we investigated what the contribution of bacteria to the *Artemia* diet is at different degressive algae feeding rations.

II. MATERIALS AND METHODS

Experimental design

Artemia were reared on four different microalgae Tetraselmis sp. rations under laboratory conditions during 15 days (Table 1). The nitrogen generated from *Artemia* waste in the cultures was converted to bacterial cells by manipulating the C/N ratio at 10 (Avnimelech, 1999). Based on the algae feeding regimes (Table 1), the nitrogen content in the algae supplied per day was used to calculate the concentration of dissolved organic nitrogen in the culture medium, assuming that the daily nitrogen concentration was approximately 50% of the feed nitrogen flux (Avnimelech, 1999). Therefore, using these nitrogen concentrations, sucrose was added once per day to produce C/N ratio 10. To assess the utilization of heterotrophic bacteria by *Artemia*, 15N NaNO3 was daily used as a tracer at 0.1% of total organic nitrogen in the culture medium to label the bacteria (Burford *et al*., 2004; Avnimelech and Kochba, 2009). Each of four feeding regimes was conducted with three replications.

Table 1: Experimental set up; *Artemia* was reared during 15 days on *Tetraselmis* sp. with four different feeding rations, and sucrose was used as carbon source to produce C/N ratio 10. No application is denoted by dash (-).

Treatment code	Algae ration	Carbohydrate
SF1 (control 1)	standard feeding	-
SF1+S10	standard feeding	sucrose
SF1/2 (control 2)	half of standard feeding	-
SF1/2+S10	half of standard feeding	sucrose
SF1/3 (control 3)	one third of standard feeding	-
SF1/3+S10	one third of standard feeding	sucrose
SF1/4 (control 4)	one fourth of standard feeding	-
SF1/4+S10	one fourth of standard feeding	sucrose

Artemia hatching and culture procedures

Dried *Artemia* franciscana Kellogg 1906 cysts, originating from Great Salt Lake, Utah (EG type; INVE Aquaculture NV, Belgium), were hydrated in tap water for 1 h, and then the cysts shells were removed by decapsulation as described by Sorgeloos *et al*. (1977) and Marques *et al*. (2006a). Decapsulated cysts were rinsed thoroughly in FIOSW to get rid of all residual bleach. Cysts were incubated in a 1 L conical glass tube containing 800 mL FIOSW at 33 g/L salinity at 28 °C for 24 h under standardized hatching conditions (Sorgeloos *et al*., 1986). *Artemia* instar I nauplii were inoculated into 1 L conical glass tubes containing 800 mL FIOSW of 33 g/L salinity at a density of 2 nauplii/mL (Naegel, 1999).

Water pH (range 7.0-8.5) was daily adjusted by adding NaHCO3 at 0.05 g/L, and tubes were provided with aeration to ensure continuous supply of oxygen in the cultures. The experiment was carried out using white neon light illumination with photoperiod 12/12. All the tubes were kept at a temperature of 28.0 ± 0.5 °C by partial submersion in a temperature-controlled water bath.

Uneaten food and wastes from *Artemia* were daily removed by siphoning before feeding, while aeration was briefly interrupted.

Food preparation

A marine Tetraselmis sp. concentrate (Instant Algae 3600; Reed Mariculture Inc., USA) was used. The microalgae concentrate contains intact cells that are non-viable. The latter was verified by the absence of a pH change over a period of 6 h with continuous illumination (± 41 µE/m2s) at an algae concentrate density of 1 g/L. As algae were metabolically non-active it is

assumed that the nitrate assimilation in the experiments was done by the bacteria. The microalgae concentrate was diluted in 0.2 µm filtered Instant Ocean artificial seawater (FIOSW) at 33 g/L salinity. The concentration of algae in the solution was measured by a Bürker counting chamber. The algal solution was stored at 4 °C for subsequent use and the number of cells, administered once daily in the morning, was increased per day according to the age of *Artemia* (Table 2)

Table 2: Feeding schedule for *Artemia* fed on microalgae (adapted from Naegel, 1999).

Day	Tetraselmis (106 cells/animal/day)
1	0.04
2	0.14
3	0.18
4	0.25
5	0.38
6	0.50
7	0.75
8	0.88
9	0.90
10-14	0.90

Data collection and sample analysis

Pre-sampling treatment

At the end day of the experiment *Artemia* were harvested and transferred to 1 L beakers containing 500 mL of FIOW and 20 µm cellulose particles (Sigma) at a concentration three times the algae cell density in the ST feeding regime, for gut evacuation. During the evacuation period, aeration was provided continuously to ensure homogeneous distribution of the cellulose in the water. *Artemia* were checked regularly for the ingestion status under a binocular microscope. Sampling for analysis was done when the digestive tract of the *Artemia* were filled completely with cellulose.

Growth of *Artemia*

Thirty animals from each replicate were randomly collected and fixed with Lugol´s solution. The individual length of *Artemia* was determined (from the front of the head to the end of the telson) using a dissecting microscope with a drawing mirror (Marques *et al*., 2004b), and by conversion to real length using the software *Artemia* 1.0® (courtesy of Marnix Van Damme)

Survival and total biomass production

Artemia were harvested at DAH15 and rinsed several times in de-mineralized water (DEMI-water) on a sieve to remove un-eaten food and waste; then *Artemia* was placed on tissue paper to remove all excess water.

Survival in each replicate was calculated according to the following equation:

Survival (%) = (final number of *Artemia*/initial number of *Artemia*) x 100

Total biomass production (TBP) in wet weight (g/L) in each tube was determined by weighing the total production (including the *Artemia* sampled for length measurement) and the average per treatment was calculated.

After obtaining survival and TBP data, sampling for 15N accumulation and fatty acid analysis in *Artemia* was done.

Nitrogen accumulation in *Artemia*

Ten cellulose-treated *Artemia* individuals from each tube were sampled randomly at DAH15 for 15N analysis. After sampling, *Artemia* were first immersed in a benzocaine solution (Sigma, 0.1%) for 10 s, transferred to a benzalkonium chloride solution (Sigma, 0.1%) for another 10 s to kill attached bacteria on their exoskeleton (Chládková *et al*., 2004), and then washed in DEMI-water to remove salt. Each sample was then put into a pre-weighed tin capsule cup (5 x 8 mm), oven-dried at 70 °C for a day (De Troch *et al*., 2007), and then cooled down in a desiccator. The dry weight of the samples was determined using a digital precision balance (precision 0.1 mg), and the level of 15N excess in *Artemia* was determined using an elemental analyzer (ANCA-SL, PDZ

Europa, UK) coupled to a continuous flow isotope-ratio mass spectrometer (CF-IRMS) (20-20, SerCon, UK) at the Department of Applied Analytical and Physical Chemistry, Ghent University, Belgium.

Nitrogen derived from heterotrophic bacteria in *Artemia* was calculated according to the formula as described by Fry (2006): the nitrogen stable isotope contents in *Artemia* are expressed as δ values in parts per thousand (‰).

δ 15N ‰ = [(Rsample – Rstd)/Rstd] x 1000

where R = ratio 15N/14N. Rstd = 0.0036765, the internationally recognized standard for atmospheric N2.

Fatty acids composition of *Artemia* and feed sources

After weighing the biomass and after taking animals for 15N, *Artemia* from each culture vial were frozen at − 20 °C for fatty acid analysis. Fatty acid methyl esters (FAME) of *Artemia* were prepared by transesterification for gas chromatography and identified by a gas chromatograph (GC), via a procedure modified from Lepage and Roy (1984) and Coutteau and Sorgeloos (1995). Briefly, 0.2 g of *Artemia* biomass was weighed on the bottom of a 35 mL glass tube with a teflon® lined screw cap. Total lipids were extracted from *Artemia* with a solvent mixture including 100 µL of internal standard solution (containing 4.78255 mg/mL 20:2n-6 or 14.39986 mg 22:2n-6 fatty acid dissolved in iso-octane), 5 mL of methanol/toluene (3:2 v/v) solution and 5 mL of freshly prepared acetylchloride/methanol (1:20 v/v) solution. The air in the tube was flushed out by nitrogen gas and the tube was then closed tightly. The product in the tube was mixed by shaking and the reaction was left to take place for 1 h at 100 °C in a boiling bath with shaking every 10 min. Then the sample was allowed to cool down and 5 mL of hexane and 5 mL of distilled water were added to the tube. The sample was extracted by centrifugation (± 2,000 x g; 5 min) with hexane and transferred into another glass tube. The combined hexane phase was dried by vacuum filtering in a 50 mL pre-weighed pear-shaped flask over a 4 cm diameter P3 filter, filled for one third with anhydrous sodium sulfate powder. The tube and the filter were rinsed several times with hexane (± 5 mL) until the flask was filled up. The solvent was evaporated on a rotary evaporator at 35 °C, flushed to dryness with nitrogen gas, and the pear-shaped flask was weighed again. The dried FAME was finally dissolved in 0.5 mL iso-octane and transferred into a 2 mL glass vial with teflon® lined screw cap. The vial was flushed with nitrogen and the sample was stored at − 30 °C until injection. For the actual GC analysis, 0.25 µL of the iso-octane dilution was injected, containing ± 2 mg FAME/mL. The individual FAME-amounts were calculated using the known amount of the internal standard as a reference.

Quantitative determination was done by a Chrompack CP9001 gas chromatograph equipped with a CP9010 liquid autosampler and a temperature-programmable on-column injector. Injections were performed on-column into a 50 m long polar capillary column, BPX70 (forte-series, SGE Australia), with a diameter of 0.32 mm and a layer thickness of 0.25 µm. The BPX70 was connected to a 2.5 m long methyl deactivated pre-column. The carrier gas was hydrogen, at a pressure of 100 kPa using a flame ionization detector (FID). The oven was programmed to rise from the initial temperature of 85 °C to 150 °C at a rate of 30 °C/min, from 150 °C to 152 °C at 0.1 °C/min, from 152 °C to 172 °C at 0.65 °C/min, from 172 °C to 187 °C at 25 °C/min and set to stay at 187 °C for 7 min. The injector was heated from 85 °C to 190 °C at 5 °C/sec and was set to stay at 190 °C for 30 min.

Analog to digital (A/D) conversion of the FID signal and subsequent data capture to a computer was done with an Agilent 35900E A/D converter. Peak identification was based on GLC-68 series standard reference mixtures, complemented by individual standards (both from Nu-Chek-Prep, Inc., USA). Integration and calculations were done on a Microsoft -Windows© -based computer using Agilent GC Chemstation Rev. B.02.01 (build 244), complemented by two custom designed Microsoft Excel© macros.

Relative survival increase and relative total biomass production increase

The relative survival increase (RSI) and relative total biomass production (TBP) increase (RTBPI) were calculated to assess the effect of carbohydrate addition to the *Artemia* culture according to the following formula:

RSI or RTBPI (%) = (survival or TBP of treatmenti – survival or TBP of controli)/ survival or TBP of controli

Where i=1-4

Statistical analysis

The data of biomass production, fatty acid levels and the values of 15N nitrogen were checked for normal distribution and homogeneity of variance by p-p plots and Levene´s test of Statistica 7.0 software for windows. If one of these assumptions could not be satisfied, data were transformed, prior to two-way analysis of variance (ANOVA), which was used to test the effect of

feeding regime and carbohydrate addition, and the interaction between these factors, on the survival, individual length and biomass production of *Artemia*. This was followed by Tukey´s honestly significant difference (HSD) test, employed at 0.05 probability level. A non-parametric, Kruskal-Wallis, test was used when the transformation could not be applied. One-way ANOVA was performed for fatty acid levels and 15N accumulation in the *Artemia* tissue.

III. RESULT

Artemia performance

Feeding regime and carbon addition both had a significant effect ($p < 0.05$) on *Artemia* survival and biomass production. Feeding regime also had a significant effect on growth ($p < 0.05$). Interaction between both variables was only significant for growth (Table 3).

Table 3: Probability levels of two-way analysis of variance (with feeding regime and carbon addition as independent factors) on survival, individual length (IL) and total biomass production (TBP) of *Artemia*. Asterisk (*) indicates significance ($p < 0.05$).

Source	p-value		
	Survival	IL	TBP
Feeding regime (FR)	0.008*	0.000*	0.000*
Carbon addition	0.000*	0.582	0.000*
FR x carbon addition	0.814	0.001*	0.491

Survival of solely microalgae-fed *Artemia* didn't strictly decrease in the order of the reduction of algae supply. When comparing to the SF1 regime, the survival of *Artemia* fed the SF1/2 regime increased, but decreased for the SF1/3 and SF1/4 regimes. In comparison to the non-treated respective controls, the addition of carbohydrate improved survival of *Artemia* in all feeding regimes, and the increase was significant ($p < 0.05$) for SF1/3, as illustrated as well by the high RSI values, 182%, for this treatment. When comparing the four feeding regimes, the best survival was shown by SF1/2+S10, SF1+S10 and SF1/3+S10, while the poorest survival was shown by SF1/4 (Table 4).

The availability of microalgae as monodiet in the culture medium affected the growth of *Artemia*: with the exception of SF1/3, the individual length gradually but non-significantly decreased when the feeding regime was gradually reduced from SF1 to SF1/4. With addition of carbohydrate, a higher individual length of *Artemia* was shown in both SF1 and SF1/2 treatments as compared to their matching control treatment, but a lower body length in case of SF1/3 and SF1/4. In the case of SF1/3 this decrease was significant ($p < 0.05$).

As a combination of growth and survival, also the TBP of *Artemia* was related to the density of algae in the culture medium. TBP gradually reduced from the SF1 to the SF1/4 regime of solely algae fed *Artemia*, and the reduction was significant in the lowest feeding treatment (SF1/4) as compared to SF1 ($p < 0.05$). The addition of carbohydrate to the *Artemia* cultures improved biomass production in all feeding regimes as compared to its corresponding control treatments, but the increase was not significant ($p > 0.05$). Moreover, the RTBPI gradually increased from around 60% for SF1 to nearly 150% for SF1/4, as compared to the matching control treatments. When comparing the four feeding regimes, the best TBP was shown by SF1+S10 and SF1/2+S10, while the poorest TBP was exhibited by SF1/4. The addition of sucrose to SF1/2 and SF1/3 feeding regimes increased the TBP of those treatments to a level equal or exceeding that obtained in the SF1 control.

Table 4: Final survival (%), individual length IL (mm) and biomass production TBP in wet weight (g/L) of *Artemia* fed on different algal paste rations and with stimulation of bacterial growth by carbohydrate addition. The values are mean ± standard deviation (n = 3). RSI = relative survival increase (%) of carbohydrate treatments as compared to respective controls; RTBPI = relative total biomass production increase (%) of carbohydrate treatments as compared to respective controls. Different superscript letters in the same column denote significant differences ($p < 0.05$). For abbreviation of the treatments, see Table 1.

Treatment code	Survival (%)	IL (mm)	TBP (g/L)	RSI (%)	RTBPI (%)
SF1 (control 1)	36.4 ± 12.7^{ab}	7.0 ± 1.6^{bc}	2.8 ± 0.6^{cd}	-	-
SF1+S10	62.4 ± 25.8^{bc}	7.9 ± 1.7^{c}	4.6 ± 1.7^{d}	84.3 ± 63.0^{ab}	60.2 ± 35.1a
SF1/2 (control 2)	41.1 ± 13.4^{abc}	6.7 ± 1.1^{abc}	2.1 ± 0.2^{abc}	-	-
SF1/2+S10	75.9 ± 5.3^{c}	6.9 ± 1.1^{abc}	3.5 ± 0.7^{cd}	54.8 ± 10.9^{a}	71.8 ± 32.7a
SF1/3 (control 3)	22.7 ± 4.1^{a}	7.2 ± 1.4^{bc}	1.2 ± 0.3^{ab}	-	-
SF1/3+S10	61.1 ± 11.6^{bc}	5.9 ± 1.1^{a}	2.7 ± 0.3^{bc}	181.6 ± 53.7^{b}	118.9 ± 25.6^{a}
SF1/4 (control 4)	14.4 ± 8.1^{a}	6.4 ± 1.3^{ab}	0.6 ± 0.3^{a}	-	-

SF1/4+S10	42.0 ± 7.6^{abc}	6.1 ± 1.3^{ab}	1.4 ± 0.3^{ab}	192.9 ± 53.3^{b}	± 99.0^{a}

Fatty acids composition of feed and *Artemia*

Dietary fatty acids composition

Results of fatty acid analysis showed that the microalgae concentrate, Tetraselmis sp., was abundant with polyunsaturated fatty acids (PUFA) (57.1 mg/g DW), but contained low levels of monounsaturated fatty acids (MUFA) (19.7 mg/g DW); particularly, both 16:1n-7 and 18:1n-7 were present in equal amounts at 3.2 mg/g DW. In contrast to the algal concentrate, bacteria contained high levels of MUFA (37.7 mg/g DW). Particularly the 16:1n-7 and 18:1n-7 levels in bacteria were respectively 5 and 2 times higher than in the microalgae concentrate. Bacteria contained minor PUFA contents, which was 21 times lower than the PUFA contents in the microalgae concentrate. Additionally, 18:3n-3 in bacteria was 10 times lower than in the microalgae concentrate, and 20:5n-3 was below detection level (Table 5.5).

Fatty acids composition of *Artemia*

The MUFA levels (mg/g DW) of *Artemia* fed solely microalgae were gradually reduced as the algal ration was reduced from the SF1 to the SF1/4 regime, resulting in a value for the SF1/4 regime significantly lower than in SF1 ($p < 0.05$). The levels of 16:1n-7 were lower in the control treatments with reduced algal ration than in SF1, but the decrease was non-significant ($p > 0.05$). The addition of carbohydrate increased MUFA levels in all feeding regimes as compared to the respective control treatments, and in case of SF1/3 and SF1/4 this increase was significant ($p < 0.05$) (Table 5). For 16:1n-7 this increase was always significant; for total MUFA and 18:1n-7 it was significant for the lowest feeding regimes SF1/3 and SF1/4 ($p < 0.05$). In the latter two feeding regimes 18:1n-7 levels increased after carbohydrate addition to levels higher than in SF1 and SF1/2.

The PUFA content (mg/g DW) in *Artemia* fed solely algae non-significantly decreased from SF1 to the other algal feeding rations. The addition of carbohydrate into the *Artemia* cultures non-significantly reduced PUFA levels as compared to the respective control treatments ($p > 0.05$). When comparing the four feeding regimes, significant difference was only found between SF1/3+S10 (19.7 mg/g DW) and the SF1 control (27.5 mg/g DW) ($p < 0.05$). Moreover, 18:3n-3 levels of PUFA in *Artemia* fed solely algae was lower in the feeding rations SF1/2, SF 1/3 and SF1/4 as compared to SF1. This fatty acid was non-significantly reduced when carbohydrate was added (except for SF1/2 where the level was constant) ($p > 0.05$). The addition of carbohydrate non-significantly reduced 20:5n-3 levels in the lowest feeding regimes (SF 1/3 and SF1/4).

Table 5: Fatty acids composition (mg/g DW) of Tetraselmis sp. concentrate, bacteria biomass (grown on sucrose) and *Artemia* biomass. Fatty acids, discussed in the text, are highlighted in grey. The values are mean ± standard deviation (n = 3). Different superscript letters in the same column denote significant differences ($p < 0.05$). MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; total FA: total fatty acids. Value below detection limits is denoted by dash (−). For abbreviation of the treatments, see Table 1.

Fatty acids	*Tetraselmis* sp.	Bacteria	SF1	SF1+S10	SF1/2	SF1/2+S10	SF1/3	SF1/3+S10	SF1/4	SF1/4+S10
14:0	1.1 ± 0.1	1.9	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.4 ± 0.2	0.8 ± 0.2	0.5 ± 0.1	0.7 ± 0.3
14:1n-5	0.2 ± 0.0	1.5	0.4 ± 0.1^{ab}	0.7 ± 0.2^{b}	0.2 ± 0.1a	0.5 ± 0.1^{ab}	0.2 ± 0.1a	0.6 ± 0.2^{ab}	0.3 ± 0.1^{ab}	0.4 ± 0.1^{ab}
15:0	0.1 ± 0.0	0.1	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
15:1n-5	-	0.1	-	-	-	-	-	0.2 ± 0.2	0.2 ± 0.0	0.8 ± 0.3
16:0	19.9 ± 1.0	9.3	12.5 ± 1.7^{b}	12.0 ± 0.6^{b}	9.9 ± 0.0^{ab}	10.1 ± 0.5^{ab}	9.0 ± 3.0^{ab}	8.9 ± 0.8^{a}	9.4 ± 0.7^{ab}	8.5 ± 1.2^{a}
16:1n-7	3.2 ± 0.2	16.0	1.4 ± 0.3^{a}	3.4 ± 1.8^{b}	0.9 ± 0.0^{a}	2.9 ± 0.9^{b}	1.0 ± 0.4^{a}	4.0 ± 1.1^{b}	1.0 ± 0.0^{a}	2.7 ± 0.8^{b}
17:0	2.5 ± 0.2	0.1	0.9 ± 0.1	1.0 ± 0.2	0.6 ± 0.0	0.8 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.7 ± 0.0	0.8 ± 0.1
17:1n-7	1.3 ± 0.0	0.4	0.4 ± 0.1	0.1 ± 0.2	0.4 ± 0.0	-	0.3 ± 0.0	0.3 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
18:0	0.6 ± 0.0	0.9	6.0 ± 0.3	6.2 ± 0.3	6.1 ± 0.0	6.1 ± 0.3	5.7 ± 1.0	5.9 ± 0.5	6.1 ± 0.1	5.9 ± 1.3
18:1n-9	10.3 ± 0.3	1.3	13.2 ± 1.8	12.9 ± 0.7	11.7 ± 0.0	11.2 ± 0.5	10.9 ± 3.1	9.4 ± 0.5	10.5 ± 0.1	9.1 ± 0.9
18:1n-7	3.2 ± 0.2	7.1	6.5 ± 0.5^{a}	7.7 ± 1.5^{abc}	6.6 ± 0.0^{a}	7.8 ± 1.0^{ab}	6.4 ± 1.6^{a}	10.1 ± 0.6^{c}	6.1 ± 0.0^{a}	9.1 ± 0.5^{bc}
18:2n-6-t	-	0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	-	0.1 ± 0.1
18:2n-6-c	8.4 ± 0.3	0.1	6.3 ± 0.9	5.6 ± 0.6	5.5 ± 0.0	5.1 ± 0.2	5.0 ± 1.2	4.3 ± 0.3	5.2 ± 0.2	4.5 ± 0.3
19:0	0.1 ± 0.0	-	-	-	-	-	-	-	-	-
18:3n-6	4.6 ± 0.3	0.6	1.9 ± 0.3	1.9 ± 0.2	1.4 ± 0.0	1.7 ± 0.1	1.3 ± 0.4	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
19:1n-9	0.1 ± 0.0	0.2	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.3
18:3n-3	20.7 ± 0.9	0.2	10.1 ± 1.9^{c}	9.1 ± 1.1^{bc}	7.6 ± 0.0^{abc}	7.9 ± 0.4^{bc}	7.4 ± 1.7abc	6.5 ± 0.6a	8.1 ± 0.6bcd	6.7 ± 0.9ab
18:4n-3	11.6 ± 0.5	0.1	3.6 ± 0.7c	3.8 ± 0.5c	2.6 ± 0.0^{abc}	3.0 ± 0.3^{abc}	2.5 ± 0.8ab	2.5 ± 0.2a	2.6 ± 0.3abc	2.5 ± 0.1a
20:0	-	0.1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
20:1n-9	0.8 ± 0.0	0.1	0.7 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.7 ± 0.3	0.5 ± 0.1	0.4 ± 0.1
20:1n-7	0.7 ± 0.0	0.3	-	-	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.5	0.1 ± 0.0	0.3 ± 0.2	0.8 ± 0.1
21:0	0.3 ± 0.0	-	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-6	0.2 ± 0.1	0.2	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	2.1 ± 0.1	0.1	1.8 ± 0.2	1.7 ± 0.1	2.1 ± 0.0	1.7 ± 0.1	1.8 ± 0.2	1.7 ± 0.2	1.7 ± 0.0	1.6 ± 0.2
20:3n-3	-	-	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:4n-3	0.8 ± 0.0	0.1	0.5 ± 0.1	0.5 ± 0.2	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.1
22:0	-	-	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1
20:5n-3	6.7 ± 0.2	-	2.9 ± 0.2^{b}	3.0 ± 0.1^{b}	3.0 ± 0.0^{b}	3.0 ± 0.1^{b}	2.9 ± 0.4^{ab}	2.5 ± 0.2^{a}	3.1 ± 0.3^{b}	2.8 ± 0.2ab

Table 5.5, continued

Fatty acids	Tetraselmis sp.	Bacteria	SF1	SF1+S10	SF1/2	SF1/2+S10	SF1/3	SF1/3+S10	SF1/4	SF1/4+S10
22:1n-9	-	-	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	-	-
22:1n-7	-	-	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
23:0	0.3	-	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	-	-
21:5n-3	-	-	-	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
23:1n-9	-	-	-	-	-	-	-	-	-	-
22:4n-6	0.5	0.3	-	-	-	-	-	-	-	-
22:3n-3	-	-	-	-	-	-	-	-	-	-
22:5n-6	-	0.1	-	-	-	-	-	-	-	-
22:4n-3	-	-	-	-	-	-	-	-	-	-
24:0	-	-	-	-	-	-	-	-	-	-
22:5n-3	0.4 ± 0.0	-	-	-	-	-	-	-	-	-
24:1n-9	-	0.1	-	-	-	-	-	-	-	-
22:6n-3	1.4 ± 0.0	0.1	0.1 ± 0.0	0.1 ± 0.0	-	-	-	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
MUFA	19.7 ± 0.8	37.7	23.3 ± 1.9bc	26.0 ± 2.1c	21.1 ± 0.2abc	23.6 ± 1.9bc	20.3 ± 1.2ab	25.6 ± 2.5c	19.5 ± 0.2a	23.7 ± 0.6c
PUFA	57.1 ± 2.6	2.7	27.5 ± 1.2b	26.4 ± 1.9ab	23.9 ± 1.2ab	23.4 ± 0.9ab	21.8 ± 2.1ab	19.7 ± 1.3a	23.2 ± 1.5ab	20.2 ± 0.6ab
Total FA	101.5 ± 5.0	64.1	82.6 ± 10.5	83.4 ± 5.1	68.7 ± 0.1	74.8 ± 4.9	65.0 ± 16.0	69.4 ± 5.3	67.2 ± 2.9	69.1 ± 4.4

Nitrogen derived from heterotrophic bacteria

The natural 15N abundance in *Artemia* was as low as 10‰ (Fig. 1).The excess of 15N in *Artemia* was inversely proportional with the availability of microalgae in the culture, resulting in values significantly higher for the lowest feeding regimes as compared to the standard regime SF1 ($p < 0.05$).

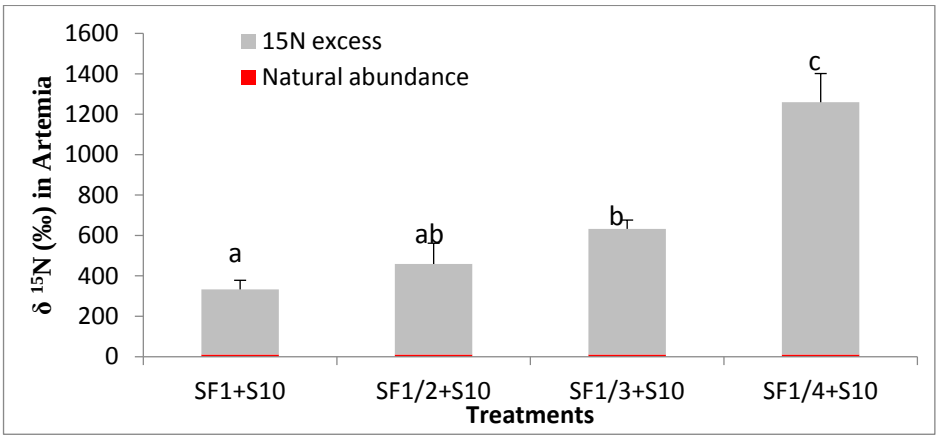

Figure 1: 15N nitrogen accumulation in *Artemia*. The values are mean ± standard deviation (n = 3). Indices a, b, c, and d are indicating homogenous subsets ($p < 0.05$). For abbreviation of the treatments, see Table 1.

IV. DISCUSSION

The survival of *Artemia* fed solely on algae concentrate at the standard feeding ration in the current study was less than 50%, which is lower than survival of *Artemia* in a previous study by Fábregas *et al*. (1996) where *Artemia* were fed live Tetraselmis during 19 days. Loss of nutritional quality may have occurred during centrifugation, harvesting, transportation and/or storage of the algae (McCausland *et al*., 1999). According to Albentosa *et al*. (1997) the process of algae preservation may affect the cell wall, hence reducing their digestibility.

This study demonstrated that stimulating bacterial growth by adding carbohydrate at C/N ratio 10 increased the survival of *Artemia* in all feeding rations. This is a confirmation of our previous finding (Toi *et al*., 2013) where manipulation of C/N ratio 10 also improved *Artemia* survival. *Artemia* fed the SF1/3 or SF1/4 regime together with sucrose addition had more or less 3-fold increase of survival (RSI values) as compared to the SF1 or SF1/2 regimes. However, the manipulation of C/N ratio 10 only improved body length of *Artemia* in SF1 and SF1/2 ration, while the body length of *Artemia* in SF1/3 and SF1/4 was reduced by sucrose addition. This finding is not entirely similar to our previous study where C/N ratio 10 addition improved body length of *Artemia* at any feeding regime. The poor growth of *Artemia* in sucrose added SF1/3 and SF1/4 treatments as compared to the corresponding control treatments may relate to the relatively high survival combined with lower food availability, due to reduced algal concentration. The nutritional supplement from the bacteria may not have been enough to compensate for the lack of nutrients from algae when compared to the matching control treatment. Moreover, the higher survival in sucrose supplemented treatments led to higher total biomass production in all sucrose added treatments as compared to the corresponding control treatments, with the most prominent (and often significant) effect at the lowest algal feeding regimes SF 1/3 and SF ¼.

The fatty acid (FA) composition in *Artemia* biomass responds to the FA composition in its diets (Zhukova *et al*., 1998). The differences in fatty acid composition between algae and bacteria allows to use certain (groups of) fatty acids as biomarkers (Chamberlain *et al*., 2005) when assessing the ingestion and utilization of bacteria by *Artemia* in combination with different algae rations (Intriago and Jones, 1993). The MUFA levels in *Artemia* in our study indicate that this group of fatty acids increases when stimulating bacterial growth in the culture medium, in agreement with previous findings (Toi *et al*., 2013). This confirms that bacteria contribute nutrients to the *Artemia* tissue. Particularly the levels of both 16:1n-7 and 18:1n-7, the main MUFA components of bacteria (Table 5), were higher in all carbohydrate supplemented treatments. These fatty acids were also found at high levels in previous studies where co-feeding of bacteria and microalgae was offered to *Artemia* (Intriago and Jones, 1993; Brown *et al*., 1996; Toi *et al*., 2013). In comparison to previous study (Toi *et al*., 2013), there was a similar trend of MUFA accumulation in *Artemia*, with a considerable increase of MUFA accumulation in *Artemia* when sucrose was administrated at low microalgae supply, and a moderate

MUFA increase when sucrose was administrated with microalgae fed ad libitum. Moreover, the stimulation of bacteria by sucrose addition didn't really influence PUFA accumulation in *Artemia*. This finding is in agreement with our previous study.

The 15N used to tag heterotrophic bacteria was found to accumulate in the *Artemia* tissue. This result confirms that *Artemia* can ingest and digest heterotrophic bacteria. Moreover, the level of 15N in *Artemia* receiving a low algal feeding regime was higher than in a high feeding regime, which is in agreement with our previous study (Toi *et al*., 2013) and which confirms that *Artemia* mostly utilized algae when algae are applied in optimal density. When comparing results of 15N versus MUFA accumulation in *Artemia*, there was dramatically gain of 15N in *Artemia* with degressive algal rations from SF1+S10 to SF1/4+S10. While the gain of MUFA in *Artemia* did not increase dramatically from SF1+S10 to SF1/4+S10.

In conclusion, the contribution of bacteria to the *Artemia* diet is clearly illustrated by this study. This bacterial contribution starts becoming substantial when the nutrients provided by microalgae are reduced to sufficiently low levels (33% and lower in this study). In these feeding conditions the TBP resulting from carbohydrate supplementation may achieve levels equal to those obtained in *Artemia* receiving a standard, exclusively algal based, feeding regime.

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