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Bacteria contribute to *Artemia* nutrition in algae-limited conditions: A laboratory study

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ABSTRACT

We investigated the effect of the stimulation of bacterial growth on *Artemia* performance in combination with a standard and with a low algal feeding regime. In both regimes, organic carbon (supplied as sucrose or soluble potato starch) and ^{15}N labeled inorganic nitrogen (supplied as $NaNO_3$) were used to stimulate bacterial growth in the *Artemia* cultures at C/N ratio 10 and 50. After a culture period of 15 days, significantly improved biomass production was obtained in all treatments with the low algae feeding regime, supplemented by carbohydrate addition. In addition, results of ^{15}N accumulation and fatty acid analysis in *Artemia* indicated that *Artemia* utilized more bacteria in algae-limited conditions. Our study shows that bacteria can be used as a nutrient source for *Artemia* compensating for suboptimal algae supply. In *Artemia* pond cultures, carbohydrate addition may hence potentially be used to stimulate the conversion of nitrogen waste into heterotrophic bacterial biomass. This can be converted into protein-rich *Artemia* biomass, especially when algae are in sub-optimal supply. These findings open perspectives for alternative *Artemia* pond production protocols, in addition to the present management procedures that exclusively focus on phytoplankton blooms as nutrient source to sustain dense *Artemia* populations.

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1. Introduction

Nauplii of the brine shrimp *Artemia* are the most commonly used live food in aquatic larviculture. Due to their nutritional value and size, *Artemia* nauplii, either freshly hatched or after nutritional enrichment, satisfy the nutritional requirements for early-stage fish and crustacean larvae (Sorgeloos et al., 2001). Being non-selective filter feeders, *Artemia* can feed on a wide range of diets such as micro-algae, bacteria, protozoa and small detritus particles. Fernández (2001) specified that the food size for *Artemia* metanauplii must range between 6.8 and 27.5 μm, with an optimum of about 16.0 μm. Its adults are able to ingest all particles less than 50 μm in size (D'Agostino, 1980; Dobbeleir et al., 1980). The use of bacteria, which are in the size range 0.6–3.0 μm (Palumbo et al., 1984), as food for *Artemia* has been reported by Intriago and Jones (1993). The ability of *Artemia* to graze on bacteria has further been demonstrated by studying the clearance rate when *Artemia* was fed radioactively labeled bacteria and measuring the amount of radioactivity accumulated in *Artemia* (Fernández, 2001).

Bacteria are easy to grow through administration of carbon and nitrogen (Gaudy and Gaudy, 1980), and the addition of carbohydrates into aquaculture systems has been reported to induce the conversion of nitrogen to bacterial protein (Avnimelech, 1999). Bacteria grown at high density tend to form bioflocs (Crab et al., 2007; De Schryver et al., 2008), which are conglomerates of bacteria, protozoa, algae, detritus etc. Bioflocs vary in size from 0.1 mm to a few mm (Avnimelech, 2011), and are thus of suitable size for uptake by aquaculture organisms such as Nile tilapia (*Oreochromis niloticus*) fingerlings (Avnimelech, 2007), white shrimp (*Penaeus vannamei*) from larvae to market size (Hari et al., 2004), and for fresh-water prawn (*Macrobrachium rosenbergii*) larvae (Crab et al., 2009a). The production of bioflocs induced by the addition of carbohydrates significantly increased the final survival and biomass production of these target animals. Additionally, promoting bacterial growth in aquaculture systems clearly reduced the demand of feed protein (Avnimelech, 1999; Burford et al., 2004; Crab et al., 2009b; Hari et al., 2004).

Artemia pond production of cysts and biomass is a profitable activity in solar saltworks in the Mekong Delta, Vietnam (Anh et al., 2009b; Baert et al., 1997). Thanks to its filtering feeding behavior, *Artemia* can be produced as a form of extractive aquaculture, lowering nutrient levels in aquaculture effluents and producing animal protein. The protein content of adult *Artemia* is around 50% of its dry weight (Anh et al., 2009a) and it can be used as an ingredient for shrimp feed, reducing

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the need for fish protein in shrimp culture. Traditionally, *Artemia* is cultured supplying animal wastes such as chicken manure, pig dung etc. and algae-rich green water from fertilizer ponds as food source. However, the carbon/nitrogen (C/N) ratio in *Artemia* food supplements is usually lower than the requirements needed to stimulate bacterial growth, e.g. within the range 4–8 for microalgae (Seixas et al., 2009) and 4–6 for soybean meal (Kuo et al., 2004). In biofloc production, this ratio is therefore increased by adding carbohydrates (Avnimelech, 1999; Crab et al., 2009b; Hari et al., 2006; Nootong et al., 2011). Avnimelech (1999) stated that C/N ratio 10 promotes bacterial growth. Later Asaduzzaman et al. (2008) and Hargreaves (2006) demonstrated that C/N ratio 10 or higher induces bacterial growth. According to other studies optimal biofloc production can be done at C/N ratio 15 (Schneider et al., 2005) or C/N ratio 20 (Asaduzzaman et al., 2008; Nootong et al., 2011). Furthermore, the growth of heterotrophic bacteria also depends on the source of carbohydrate supplied (Asaduzzaman et al., 2008; Kuhn et al., 2009).

In *Artemia* pond culture, quantification of the dietary contribution of bacteria is difficult to perform. Hence, in this study carbohydrate addition in an *Artemia* laboratory culture aimed to stimulate the conversion of nitrogen in the culture medium into heterotrophic bacterial biomass using different C/N ratios and carbohydrate sources. To clarify the possible positive and negative effects of bacterial growth in the culture medium and the effect of ingestion and assimilation of bacteria on *Artemia* performance, a broad range of C/N ratios was chosen in this study. We used C/N ratio 10 as lower value, because its effects on bacterial growth are relatively well documented in literature. As higher value C/N 50 was chosen as this is far above the range 10–20 described in literature. The contribution of the heterotrophic bacteria to the *Artemia* diet was assessed at different algal densities, and using *Artemia* survival, growth and total biomass production as criteria for culture success. The assimilation of bacteria was determined by the addition of ^{15}N-nitrogen into the cultures to label the bacteria (Avnimelech and Kochba, 2009; Burford et al., 2004) and subsequent measurement of the ^{15}N accumulation in *Artemia*. Moreover, as algae and bacteria are characterized by specific fatty acid profiles, and as dietary fatty acids are transferred conservatively into *Artemia* lipids (Intriago and Jones, 1993; Zhukova et al., 1998), the *Artemia* fatty acid profile was determined at the end of the culture period in order to assess the extent of assimilation of heterotrophic bacteria by *Artemia*.

2. Materials and methods

2.1. Experimental design

Artemia was cultured over a period of 15days under zero-water exchange. The different feeding regimes and different conditions stimulating bacterial growth were investigated for their effects on *Artemia* performance. *Artemia* were fed with microalgae concentrate as the main food source (see Section 2.2). From the first day after hatching (DAH1) to DAH4, *Artemia* were acclimated in identical culture conditions using a standard algal feeding (SF) regime without carbohydrate addition: preliminary tests had shown that due to the relatively low clearance rate of the youngest *Artemia* stages (Makridis and Vadstein, 1999), carbohydrate addition during this initial period resulted in quick biofloc formation due to poor uptake of bacteria by *Artemia*. From DAH5 onwards, carbohydrate was added to the cultures: the *Artemia* were split up into two groups under two different feeding regimes, standard and low (the latter being ¼ of the standard feeding regime). For each feeding regime, two different conditions of bacterial growth stimulation, C/N ratio 10 and 50, were applied. For each C/N ratio and feeding regime, two different carbon sources (sucrose and soluble potato starch) were used (Table 1). Soluble potato starch and sucrose were first dissolved in a limited amount of boiling water, left to cool down, and then provided to the *Artemia* cultures.

Table 1
Experimental set up; *Artemia* was reared over 15 days and fed on two different feeding regimes: standard feeding regime (SF) and low feeding regime (LF). C/N: carbon/nitrogen; S: sucrose; ST: soluble potato starch. No application is denoted by dash (–).

Treatment code	Algae ration		Carbon source	C/N ratio
	Days 1–4	Days 5–14		Days 5–14
1. SF (control 1)	SF	SF	–	5.7
2. SF + S10	SF	SF	Sucrose	10
3. SF + ST10	SF	SF	Soluble potato starch	10
4. SF + S50	SF	SF	Sucrose	50
5. SF + ST50	SF	SF	Soluble potato starch	50
6. LF (control 2)	SF	LF[a]	–	5.7
7. LF + S10	SF	LF	Sucrose	10
8. LF + ST10	SF	LF	Soluble potato starch	10
9. LF + S50	SF	LF	Sucrose	50
10. LF + ST50	SF	LF	Soluble potato starch	50

[a] LF = ¼ of SF.

C/N ratio calculation was based on a protein content of 54.66% for the *Tetraselmis* sp. concentrate used (information provided by Reed Mariculture Inc., USA) and a conversion factor to nitrogen of 1/6.25 for algae (Lourenço et al., 1998). Furthermore, as the carbon content of algae can be considered as around 50% (Behrens, 2005), the C/N ratio of the algae diet (which is approximately 5.7; information provided by Reed Mariculture Inc., USA), was lower than the optimum for subsequent complete N assimilation by bacteria. $NaNO_3$ was used as inorganic nitrogen source for all treatments (except for the controls) following the equation below:

$$\text{N needed (mg) per day} = \text{algae N content in SF (mg)} - \text{algae N content in LF (mg)}.$$

The carbon sources and inorganic nitrogen were daily adjusted according to the feeding regime (Table 2). ^{15}N–$NaNO_3$ was added into the *Artemia* cultures (except for the controls) once daily at a concentration of 0.1% of total nitrogen in the culture medium and in the diet to label bacteria (Burford et al., 2004). Each treatment was conducted in three replicates.

2.2. 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 pH change over a period of 6 h with continuous illumination ($\pm$41 μE/m^2 s) 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

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

Day	*Tetraselmis* (10^6 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

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).

2.3. 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 cyst shells were removed by decapsulation as described by Sorgeloos et al. (1977) and Marques et al. (2006). 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 $NaHCO_3$ 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.

2.4. Data collection and sample analysis

2.4.1. 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* was filled completely with cellulose.

2.4.2. 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., 2004), and by conversion to real length using the software *Artemia* 1.0® (courtesy of Marnix Van Damme).

2.4.3. 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:

$$\text{Survival } (\%) = (\text{final number of } \textit{Artemia}/\text{initial number of } \textit{Artemia}) \times 100.$$

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 ^{15}N accumulation and fatty acid analysis in *Artemia* was done.

2.4.4. Nitrogen accumulation from heterotrophic bacteria

Ten cellulose-treated *Artemia* individuals from each tube were sampled randomly at DAH15 for ^{15}N 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 × 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 ^{15}N 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 (‰).

$$\delta^{15}N\ ‰ = \left[\left(R_{sample} - R_{std}\right)/R_{std}\right] \times 1000$$

where R = ratio $^{15}N/^{14}N$. $R_{std} = 0.0036765$, the internationally recognized standard for atmospheric N_2.

2.4.5. Fatty acid analysis

After weighing the biomass and after taking animals for ^{15}N, *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 20:2(n − 6) or 14.39986 mg 22:2(n − 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 (4000 rpm; 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/s 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.

2.5. Statistical analysis

Statistical analysis was performed using Statistica 7.0 for Windows. The data were first checked for homogeneity of variance and normality of distribution by Levene's *F* test and the P–P plot, respectively. The data of fatty acids, ^{15}N nitrogen and biomass production failed to meet these assumptions and were logarithmically transformed in order to satisfy normal distribution and to homogenize variance. For the same reason survival data were square root transformed. For all datasets, one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post-hoc test at 0.05 probability level was employed.

3. Results

3.1. Artemia performance

The feeding regime significantly affected the survival of *Artemia* (Table 3). Survival in SF-fed *Artemia* was significantly higher than in LF-fed *Artemia* ($P<0.05$). The addition of carbohydrate from DAH5 onwards did not have a consistent effect on the survival of *Artemia*. Under the SF regime, addition of carbohydrate at C/N ratio 50 produced a lower survival than the control for both carbohydrate sources, but the decrease was not significant ($P>0.05$). In contrast, addition of starch at C/N ratio 10 resulted in higher survival, though not significantly ($P>0.05$), than in the control, while addition of sucrose at C/N ratio 10 produced a similar result as the control.

Under the LF regime, all treatments resulted in better survival than the control, but the increase was not significant ($P>0.05$). In comparison, all lower algal feeding treatments receiving carbon supplementation resulted in a survival similar to the value obtained by standard algal feeding ($P>0.05$), except for the standard algal feeding treatment receiving ST at C/N ratio 10.

The feeding regime also significantly affected the growth of *Artemia*. Under the SF regime, the growth was non-significantly stimulated in all treatments, except for ST 50 where growth was non-significantly slowed down ($P>0.05$). In contrast to the SF regime, addition of both carbon sources at both C/N ratios produced better growth in LF-fed *Artemia*, and except for ST at C/N ratio 10 the increase was significant ($P<0.05$).

As a net result of survival and length, biomass production was not significantly changed by carbohydrate addition in SF treatments. In contrast, carbohydrate addition increased more than two-fold the biomass production in all LF treatments resulting in a significant difference between those treatments and the LF control ($P<0.05$).

Table 3
Survival (%), individual length (mm) and biomass production (g/L) of *Artemia* fed two different feeding regimes and different culture conditions stimulating bacterial growth. Values are mean ± standard deviation (n = 3). Different superscripts in the same column denote significant differences ($P<0.05$). For abbreviations of treatments, see Table 1.

Treatment code	Survival (%)	Individual length (mm)	Biomass production (g/L)
1. SF (control 1)	39.2 ± 4.6^{b}	7.6 ± 1.2^{de}	4.4 ± 0.1^{de}
2. SF + S10	38.6 ± 6.2^{ab}	8.2 ± 1.6^{e}	5.1 ± 0.1^{e}
3. SF + ST10	46.9 ± 6.1^{b}	8.3 ± 1.4^{e}	5.4 ± 0.5^{e}
4. SF + S50	28.7 ± 12.0^{ab}	8.0 ± 1.5^{e}	3.6 ± 1.2^{cde}
5. SF + ST50	29.3 ± 2.3^{ab}	6.9 ± 1.1^{cd}	2.6 ± 0.1^{bcd}
6. LF (control 2)	21.9 ± 6.2^{a}	5.1 ± 1.3^{a}	1.0 ± 0.1^{a}
7. LF + S10	33.7 ± 2.8^{ab}	6.6 ± 1.1^{bc}	2.3 ± 0.2^{bc}
8. LF + ST10	33.3 ± 2.8^{ab}	5.7 ± 0.9^{ab}	2.0 ± 0.2^{b}
9. LF + S50	37.7 ± 9.2^{ab}	6.3 ± 0.9^{bc}	2.3 ± 0.6^{bc}
10. LF + ST50	30.8 ± 6.7^{ab}	6.6 ± 1.0^{bcd}	2.1 ± 0.2^{b}

3.2. Fatty acid composition of food and Artemia

3.2.1. Fatty acid composition of algae

The biochemical analysis of *Tetraselmis* sp., as food for *Artemia*, showed that the level of polyunsaturated fatty acids (PUFA) was almost three times higher than that of monounsaturated fatty acids (MUFA). Especially 18:3n − 3 and 20:5n − 3 levels were around seven times and two times higher than both 16:1n − 7 and 18:1n − 7, respectively (Table 4).

3.2.2. Fatty acid composition of Artemia

Fatty acid analysis of *Artemia* sampled at the end of the culture period showed that there were differences in PUFA and MUFA levels in some of the LF and some of the SF treatments, but the difference was not always significant (Table 4). When carbohydrate was added to the *Artemia* cultures, the total MUFA increased at both feeding regimes as compared to the respective controls. The MUFA increase was always significant for the LF treatments, while it was always non-significant for the SF treatments, except for the S50 treatment where the MUFA level was significantly higher than in the SF control. In the MUFA fraction, the addition of carbohydrate increased 16:1n − 7 and 18:1n − 7 levels in both feeding regimes, and the increase was always significant in the LF regime. Especially in the LF regime the increase of these two fatty acids was relatively higher than in the SF regime; e.g. the 16:1n − 7 level in the LF treatments was 8 to 10 times higher than in the LF control, while it was only 2.5 to 7 times higher in the SF treatments than in the SF control, except for ST10.

In addition, the total PUFA of *Artemia* did not significantly change as compared to the control in the SF regime receiving carbohydrate addition, except for a significantly lower PUFA value after the addition of ST at C/N ratio 50 ($P<0.05$). In contrast, carbohydrate addition to LF treatments always caused a reduction in PUFA level as compared to the control, but this decrease was not significant ($P>0.05$), except for ST50 (Table 4). In the PUFA fraction, except for S10 in the SF regime, 18:3n − 3 of *Artemia* was always lower after carbohydrate addition as compared to the respective control, though the decrease was only significant in ST50. Carbohydrate addition did not change the 20:5n − 3 level of *Artemia* under the SF regime, while the carbohydrate addition significantly reduced the 20:5n − 3 level of *Artemia* in almost all treatments of the LF regime (Table 4).

As for total FA, under the SF regime, FA levels were significantly higher with the addition of sucrose at C/N ratio 50 than the control ($P<0.05$), while FA levels were lower with starch addition at C/N ratio 50 than the control, but the difference was non-significant. Under the LF regime, carbohydrate addition always resulted in higher FA levels than the control, but the increase was only significant ($P<0.05$) when sucrose was added at C/N ratio 50. Addition of carbohydrate enhanced FA in *Artemia* fed low feeding regimes to values similar to the control of the standard feeding regime.

Table 4
Fatty acid composition (mg/g DW) of *Tetraselmis* sp. paste and of *Artemia* fed two different feeding regimes and using different culture conditions stimulating bacterial growth. Values are mean ± standard deviation (n = 3). Different superscripts in the same row denote significant differences (P<0.05). Rows without superscripts had no significant differences among values. MUFA: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids; Total FA: total fatty acids. For abbreviations of treatments, see Table 1. Results below the detection limit are indicated by dash (–).

Fatty acids	*Tetraselmis* sp.	SF (control 1)	SF + S10	SF + ST10	SF + S50	SF + ST50	LF (control 2)	LF + S10	LF + ST10	LF + S50	LF + ST50
14:0	1.1 ± 0.1	0.6 ± 0.1^{ab}	0.9 ± 0.1^{b}	0.7 ± 0.1^{ab}	1.6 ± 0.1^{d}	0.6 ± 0.1^{ab}	0.4 ± 0.0^{a}	1.0 ± 0.2^{bc}	0.8 ± 0.3^{b}	1.3 ± 0.1^{cd}	0.8 ± 0.0^{b}
14:1n − 5	0.2 ± 0.0	0.3 ± 0.0^{ab}	0.6 ± 0.2^{b}	0.4 ± 0.1^{ab}	0.3 ± 0.0^{ab}	0.3 ± 0.1^{ab}	0.2 ± 0.0^{a}	0.1 ± 0.1^{a}	0.5 ± 0.3^{b}	0.2 ± 0.1^{ab}	0.4 ± 0.1^{ab}
15:0	0.1 ± 0.0	0.3 ± 0.0^{abc}	0.4 ± 0.0^{c}	0.3 ± 0.0^{bc}	0.3 ± 0.1^{abc}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.0^{ab}	0.2 ± 0.1^{a}	0.3 ± 0.0^{bc}	0.2 ± 0.0^{ab}
15:1n − 5	–	–	0.1 ± 0.1	0.1 ± 0.1	–	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.2	–	–	0.5 ± 0.1
16:0	19.9 ± 1.0	11.3 ± 1.8^{abc}	13.4 ± 0.9^{cd}	11.1 ± 0.5^{abc}	15.2 ± 1.2^{d}	9.1 ± 1.0^{ab}	8.1 ± 0.3^{a}	9.9 ± 1.3^{ab}	10.2 ± 2.0^{abc}	12.0 ± 0.4^{bcd}	9.1 ± 0.5^{ab}
16:1n − 7	3.2 ± 0.2	1.4 ± 0.3^{a}	3.7 ± 0.4^{bc}	2.4 ± 0.4^{ab}	9.9 ± 0.7^{e}	5.3 ± 0.9^{cd}	0.9 ± 0.0^{a}	7.4 ± 2.3^{d}	8.7 ± 2.3^{e}	9.1 ± 0.2^{e}	9.7 ± 0.7^{e}
17:0	2.5 ± 0.2	0.7 ± 0.1^{ab}	0.9 ± 0.2^{b}	0.8 ± 0.0^{ab}	0.8 ± 0.0^{ab}	0.7 ± 0.0^{ab}	0.6 ± 0.0^{a}	0.7 ± 0.1^{ab}	0.6 ± 0.1^{a}	0.8 ± 0.1^{ab}	0.7 ± 0.0^{ab}
17:1n − 7	1.3 ± 0.0	0.5 ± 0.0^{ab}	0.7 ± 0.1^{bc}	0.6 ± 0.0^{abc}	0.6 ± 0.0^{abc}	0.4 ± 0.0^{ab}	0.3 ± 0.0^{a}	0.9 ± 0.3^{c}	0.4 ± 0.1^{ab}	0.6 ± 0.1^{abc}	0.5 ± 0.0^{ab}
18:0	0.6 ± 0.0	6.3 ± 0.8^{ab}	6.5 ± 0.5^{ab}	5.7 ± 0.1^{a}	7.3 ± 0.1^{b}	5.7 ± 0.1^{a}	6.5 ± 0.0^{ab}	6.1 ± 0.6^{ab}	6.6 ± 0.5^{ab}	6.8 ± 0.2^{ab}	6.1 ± 0.2^{ab}
18:1n − 9	10.3 ± 0.3	14.0 ± 1.6^{de}	16.1 ± 1.5^{e}	13.8 ± 0.7^{de}	13.2 ± 0.4^{cde}	8.4 ± 0.3^{a}	11.7 ± 0.5^{abc}	9.7 ± 0.8^{ab}	9.9 ± 0.7^{ab}	11.1 ± 0.6^{bc}	8.5 ± 0.2^{a}
18:1n − 7	3.2 ± 0.2	5.6 ± 0.6^{a}	7.6 ± 0.8^{ab}	5.6 ± 0.2^{a}	12.8 ± 0.7^{d}	9.7 ± 1.1^{bc}	6.2 ± 0.5^{a}	10.3 ± 1.9^{cd}	12.1 ± 1.5^{cd}	12.4 ± 1.0^{cd}	12.9 ± 0.4^{d}
18:2n − 6-t	–	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 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2
18:2n − 6-c	8.4 ± 0.3	4.6 ± 0.3^{cd}	5.0 ± 0.5^{d}	4.4 ± 0.1^{cd}	4.0 ± 0.2^{bc}	3.0 ± 0.0^{a}	4.2 ± 0.3^{cd}	3.1 ± 0.3^{ab}	3.2 ± 0.2^{ab}	3.3 ± 0.2^{ab}	2.4 ± 0.1^{a}
19:0	0.1 ± 0.0	–	–	0.1 ± 0.0	–	–	–	0.1 ± 0.0	–	–	–
18:3n − 6	4.6 ± 0.3	1.6 ± 0.3^{bcd}	1.8 ± 0.3^{d}	1.6 ± 0.1^{cd}	1.8 ± 0.2^{d}	1.3 ± 0.1^{bcd}	1.1 ± 0.1^{bcd}	1.4 ± 0.4^{bcd}	1.1 ± 0.1^{abc}	1.5 ± 0.2^{bcd}	0.9 ± 0.0^{a}
19:1n − 9	0.1 ± 0.0	0.6 ± 0.8	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.3	0.1 ± 0.0	0.6 ± 0.8	–
18:3n − 3	20.7 ± 0.9	9.3 ± 1.3^{de}	10.4 ± 0.5^{e}	9.1 ± 0.4^{de}	8.8 ± 0.3^{de}	5.8 ± 0.2^{b}	7.3 ± 0.9^{cd}	5.9 ± 0.5^{b}	5.7 ± 0.5^{b}	6.6 ± 0.3^{bc}	4.6 ± 0.1^{a}
18:4n − 3	11.6 ± 0.5	2.9 ± 0.4^{cd}	3.4 ± 0.3^{d}	3.0 ± 0.2^{cd}	3.3 ± 0.3^{d}	2.1 ± 0.2^{ab}	2.0 ± 0.3^{ab}	2.0 ± 0.2^{ab}	1.9 ± 0.1^{ab}	2.4 ± 0.3^{bc}	1.6 ± 0.1^{a}
20:0	–	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1n − 9	0.8 ± 0.0	0.8 ± 0.2^{bcd}	0.8 ± 0.2^{bcd}	0.8 ± 0.1^{cd}	1.1 ± 0.0^{d}	0.4 ± 0.0^{ab}	0.7 ± 0.1^{bcd}	0.6 ± 0.1^{abc}	0.6 ± 0.1^{abc}	0.9 ± 0.1^{cd}	0.4 ± 0.0^{a}
20:1n − 7	0.7 ± 0.0	–	–	–	–	–	0.5 ± 0.0	0.3 ± 0.3	–	0.1 ± 0.0	–
21:0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.8 ± 1.1	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n − 6	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:4n − 6	2.1 ± 0.1	1.4 ± 0.2	1.5 ± 0.4	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
20:3n − 3	–	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n − 3	0.8 ± 0.0	0.4 ± 0.0^{ab}	0.5 ± 0.0^{b}	0.4 ± 0.0^{ab}	0.8 ± 0.2^{d}	0.4 ± 0.0^{ab}	0.4 ± 0.1^{ab}	0.4 ± 0.1^{ab}	0.3 ± 0.0^{ab}	0.4 ± 0.0^{ab}	0.3 ± 0.0^{a}
22:0	–	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.1
20:5n − 3	6.7 ± 0.2	3.5 ± 0.6^{bcd}	3.6 ± 0.8^{bcd}	3.6 ± 0.2^{bcd}	3.7 ± 0.1^{d}	2.9 ± 0.1^{bcd}	3.6 ± 0.1^{cd}	2.7 ± 0.1^{b}	2.8 ± 0.2^{bcd}	2.7 ± 0.1^{b}	2.0 ± 0.1^{a}
22:1n − 9	–	–	–	–	–	–	–	–	–	–	–
22:1n − 7	–	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
23:0	0.3	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.1	0.2 ± 0.0	0.1 ± 0.0
21:5n − 3	–	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	–	0.5 ± 0.4	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
23:1n − 9	–	–	–	–	–	–	–	–	–	–	–
22:4n–6	0.5	–	–	–	–	–	–	–	–	–	
22:3n − 3	–	–	–	–	0.1 ± 0.0	–	–	–	–	–	–
22:5n − 6	–	–	–	–	0.1 ± 0.0	–	–	–	–	–	–
22:4n − 3	–	–	–	–	–	–	–	–	–	0.1 ± 0.0	–
24:0	–	–	–	–	–	0.1 ± 0.0	–	–	–	–	–
22:5n − 3	0.4 ± 0.0	–	–	–	0.1 ± 0.0	–	–	–	–	–	–
24:1n − 9	–	–	–	–	–	–	–	–	–	–	–
22:6n − 3	1.4 ± 0.0	–	–	–	0.1 ± 0.0	–	–	–	–	–	–
MUFA	19.7 ± 0.8	23.6 ± 2.5^{ab}	30.0 ± 3.0^{bcd}	24.2 ± 1.2^{ab}	38.4 ± 2.0^{d}	25.2 ± 2.5^{abc}	21.0 ± 0.4^{a}	30.1 ± 4.2^{bcd}	32.8 ± 4.6^{cd}	35.2 ± 1.8^{d}	33.3 ± 0.9^{d}
PUFA	57.1 ± 2.6	24.6 ± 3.5^{cd}	27.1 ± 2.8^{d}	24.1 ± 0.4^{cd}	24.8 ± 0.4^{cd}	17.4 ± 0.7^{b}	20.7 ± 1.6^{bc}	17.3 ± 1.0^{b}	16.9 ± 0.9^{ab}	18.8 ± 1.1^{b}	13.9 ± 0.4^{a}
Total FA	101.5 ± 5.0	75.0 ± 10.1^{abc}	89.2 ± 8.7^{cd}	75.2 ± 2.7^{abc}	98.7 ± 4.9^{d}	66.4 ± 5.1^{a}	64.4 ± 2.9^{a}	72.7 ± 6.4^{abc}	74.3 ± 9.0^{abc}	85.4 ± 6.3^{bcd}	70.0 ± 0.7^{ab}

3.3. Nitrogen derived from heterotrophic bacteria

^{15}N analysis showed that under the SF regime, the excess level of ^{15}N in *Artemia* was significantly lower in both treatments with C/N ratio 10 than in those with C/N ratio 50 (P<0.05) (Fig. 1). In the latter two, the level of ^{15}N in the sucrose treatment was significantly higher than in the starch treatment (P<0.05). Under the LF regime, the level of ^{15}N in S10 was significantly lower than in S50 (P<0.05), whereas

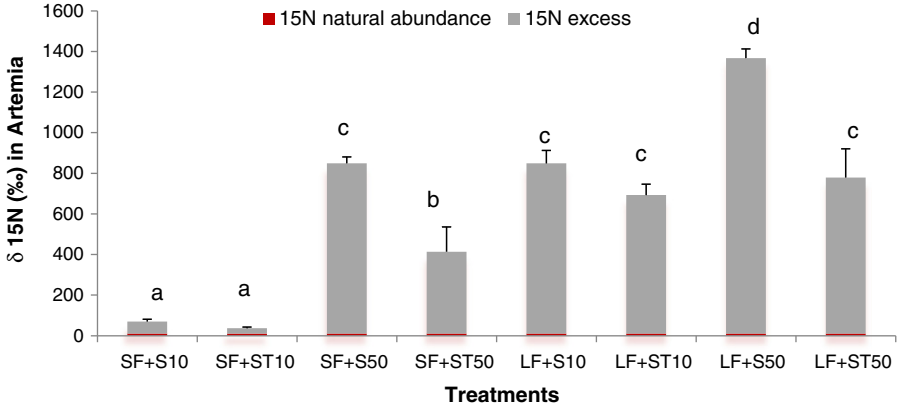

Fig. 1. Nitrogen accumulation in *Artemia* biomass fed two different feeding regimes and different culture conditions stimulating bacterial growth. Values are mean ± standard deviation (n = 3). Indices a, b, c and d are indicating homogenous subsets (P<0.05). For abbreviations of treatments, see Table 1.

the level of ^{15}N in ST10 was not significantly different from ST50 ($P > 0.05$). In the LF regime, for each C/N ratio, sucrose addition resulted in significantly higher ^{15}N excess levels than starch addition. Moreover, the excess level of ^{15}N in all LF treatments was significantly higher than in the corresponding SF treatments ($P < 0.05$).

4. Discussion

Previous studies have demonstrated that bacterial growth stimulated by carbohydrate supplementation not only improves water quality but also increases the production of target aquaculture animals (Avnimelech, 1999; Crab et al., 2009b; Hari et al., 2004; Nootong et al., 2011). Therefore, in our study, the effect of stimulation of bacterial growth on *Artemia* performance using different culture conditions was investigated. The results show that an improved *Artemia* performance in terms of body length and higher biomass yield was obtained in carbohydrate added treatments, which suggests that bacteria grown on carbohydrate contributed as nutrition source for *Artemia*. Apart from the beneficial effect of bacteria to *Artemia* growth through the contribution of extra nutrients, they are also believed to contribute with enzymes to breakdown of food (Erasmus et al., 1997; Intriago and Jones, 1993).

However, the effect obtained in different culture conditions was not the same. Under standard feeding regime, generally slow growth and poor biomass production of *Artemia* were obtained for both carbohydrate sources at the high C/N ratio as compared to the low C/N ratio and the control. This may be related to excessive bacterial growth or to the growth of nutritionally less favorable bacteria. Also biofloc formation in conditions of high C/N ratio (Asaduzzaman et al., 2008), might prevent uptake of bacteria by *Artemia*. At high densities bacteria tend to form bioflocs (Avnimelech, 1999), which could easily be observed by visual examination of the culture vials. Due to their size, in the range from 0.1 mm to a few mm (Avnimelech, 2011), they are too big for uptake by *Artemia*. Moreover, culture water viscosity was increased by massive growth of heterotrophic bacteria, which affected *Artemia* swimming activities, as demonstrated for other zooplankton (Hagiwara et al., 1998). Reduced swimming activities may be linked to reduced food utilization and to higher energy consumption for locomotion through the viscous water, resulting in retarded growth. In contrast, stimulation of bacterial growth conditions at high C/N ratio did not negatively affect *Artemia* performance at deficient algae supply; higher body length and total biomass production were obtained when low algae levels were compensated by providing carbohydrate as bacterial substrate at both C/N ratios.

Carbohydrate addition increased the total fatty acids in *Artemia* in the present study, mainly due to the increase in MUFA. The fatty acid composition of *Artemia* is correlated to its diet (Zhukova et al., 1998). Most bacteria lack PUFA, and 16:1n−7 and 18:1n−7 are major fatty acid constituents of bacteria (Intriago and Jones, 1993). These fatty acids increased in all carbohydrate added treatments, and this increase was higher at low than at high algae levels.

In contrast, the PUFA level in *Artemia* in our study reflected the algae contribution in the diet. In *Tetraselmis*, 18:3n−3 and 20:5n−3 were found as the major fatty acids (Table 4), in agreement with Bonaldo et al. (2005). The assimilation of these fatty acids in *Artemia* was not affected by carbohydrate added treatments at optimal algae supply, except for starch addition at high C/N ratio, which may be explained by bacterial proliferation, as described above, leading to biofloc formation and to higher water viscosity. Reduced assimilation of these fatty acids in *Artemia* when starch was added at high C/N ratio was also observed under low algae supply.

Besides the fatty acid composition of the *Artemia* tissue, also the ^{15}N accumulation in *Artemia* illustrated the ingestion and assimilation of bacteria by *Artemia*, as has been demonstrated for organisms of aquaculture importance by ^{15}N tagged biofloc forming bacteria (Avnimelech and Kochba, 2009; Burford et al., 2004). In our study, ^{15}N used to label the heterotrophic bacteria was found to accumulate in *Artemia*. Addition of sucrose resulted in significantly higher ^{15}N accumulation than when using starch, probably because sucrose results in the production of microbial biomass that is more easily taken up or digested by *Artemia*. Our results indicated that *Artemia* utilized more bacteria under algae-limited conditions, than under conditions of optimal algae supply. As *Artemia* is a non-selective filter feeder, probably the particle size of the food is an important factor related to the *Artemia* clearance efficiency. According to Makridis and Vadstein (1999) and Moore and Jaeckle (2010) the clearance rate (volume of cleared particles per unit of time) of larval *Artemia* on algal particles (size 12 μm) is 69 times higher than on bacterial particles (size 0.5 μm). This difference may explain why *Artemia* mostly utilized algae when algae were supplied in optimal quantities. However, the results from ^{15}N accumulation and fatty acids assimilation in *Artemia* indicate that bacteria can be utilized as a nutrient source for *Artemia* and that those bacteria may partially compensate for suboptimal algae supply. Alternatively, algae might be more digestible than bacteria. Hence, when sufficient algae are supplied together with bacteria, the gut transient time might be just sufficient for digestion of the algae while bacteria might leave the gut only partially digested. At lower algal densities the gut transit time might be longer, as less particles are taken up, allowing more time for co-ingested bacteria to be digested. At the moment, there is no sufficient scientific evidence to underpin either of the two suggested possibilities. It cannot be excluded neither that both processes (difference in clearance rate and digestibility) are both simultaneously responsible for the observations made.

In conclusion, this study demonstrates that bacteria can be used as food source for *Artemia*, especially when algal supply is limited. The nutritional quality of the in-situ produced bacteria might depend on the standing C/N ratio and/or the carbon source supplied. In *Artemia* pond production, current management procedures intensively focus on the induction of a phytoplankton bloom as food for the *Artemia* population (Anh et al., 2009b). Although our results still remain to be validated in pond production conditions, our study demonstrates that in specific conditions the bacterial flora may significantly contribute as a dietary source to *Artemia* performance, and that this is especially the case in algae-limited conditions. This opens up new perspectives for alternative protocols for pond production, in which the focus may rely more or entirely on bacteria as a source of food for the *Artemia* population.

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