Contribution of bacteria to the *Artemia* diet

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List of abbreviations and units

°C	Degree Celsius
%	Percentage
µg	Microgram
µm	Micrometre
µL	Microlitre
ω	Omega
δ	Delta
× *g*	Relative centrifugal force or G force
±	Approximately
/	Per
Δ	Isotopic discrimination
A	Analog
AFDW	Ash free dry weight
ANOVA	Analysis of variance
ASAP	Alternative Splicing Annotation Project
At	Atom
BAPNA	N-alpha-benzoyl-DL-arginine-p-nitroanilide
C	Carbon
CCAP	Culture Collection of Algae and Protozoa
CF-IRMS	Continuous flow isotope-ratio mass spectrometer
CFU	Colony forming unit
cm	Centimeter
D	Digital
DGGE	Denaturing gradient gel electrophoresis
DAH	Day after hatching

DNA	Deoxyribonucleic acid
DT	*Dunaliella tertiolecta*
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
EUROSCARF	European *Saccharomyces cerevisiae* Archive for Functional Analysis, Germany
F	Forward primer
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Food and Agricultural Organization of the United Nations
FAASW	Filtered and autoclaved artificial seawater
FID	Flame ionization detector
FIOSW	Filtered Instant Ocean seawater
FR	Feeding regime
g	Gram
GC	Gas chromatograph
h	Hour
IRMS	Isotope-ratio mass spectrometry
IL	Individual length
kPa	kilopascal
L	Liter
LVS…	Strain … isolated by Laurent Verschuere
m^2	Square meter
m^3	Cubic meter
M	Molar
MA	Marine agar 2216

MB	Marine broth 2216
M-MB	Modified marine broth
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
MUFA	Monounsaturated fatty acids
N	Nitrogen
n	Number of replicates
nm	Nanometer
no.	Number
HSD	Honestly significant difference
OD	Optical density
p	Statistical p-value obtained
PCR	Polymerase chain reaction
pH	Measure of the acidity of a solution
ppt	Parts per thousand
PUFA	Polyunsaturated fatty acids
R	Reverse primer
R_{sample}	Ratio of the concentrations (atom fraction) of the heavy and light isotopes
$R_{standard}$	Ratio of an internationally recognized standard
RNA	Ribonucleic acid
rpm	Rotations per minute
RTBPI	Relative total biomass production increase
RSI	Relative survival increase
s	Second

TBP	Total biomass production
TL	Total length
W	Watt
WT	Wild type yeast strain
YEPD	Yeast extract peptone dextrose medium
v	Volume

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Chapter 1

Introduction

Chapter 1: Introduction

1.1. Importance of aquaculture

According to United Nations estimates, the world population has reached 7 billions in October 2011. New challenges arise around the world to provide sufficient and adequate food for those people. High protein foods are a necessary requirement for good health. Fish and other aquatic products are valuable sources of high quality proteins, minerals and vitamins. Moreover, fish, especially marine fish, is rich in ω-3 polyunsaturated fatty acids (n-3 PUFA), the health benefits of which are broadly recognized (Stone, 1996). Due to growing demand, the global average fish consumption per capita per year reached 18.8 kg in 2011 (FAO, 2012). However, capture fisheries cannot deal with the growth of the world population; therefore aquaculture has expanded to meet the human demand. The total aquaculture production has increased more than 120-fold from 0.64 million tonnes in 1950 to 78.9 million tonnes in 2010 (Fig. 1.1).

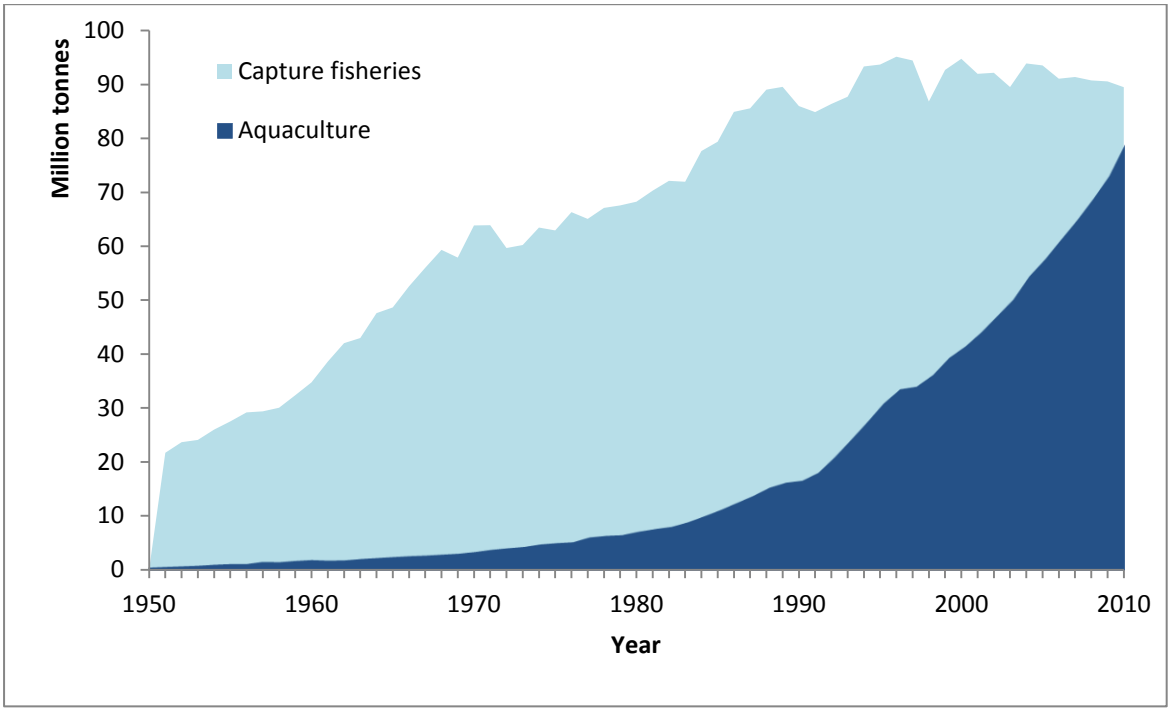

Figure 1.1: World capture fisheries and aquaculture production from 1950 to 2010 (Source: FAO, 2012)

Besides aquaculture´s contribution to food security for humans, it also contributes to the world economic growth. The world's export trade of fish and fisheries products

was worth approximately USD 55.75 billion in 2000, and it has continued growing, reaching USD 108.56 billion in 2010. It is foreseen that the trend will continue to increase and that aquaculture will be the major contributor to meet the increasing demand of the growing world population for aquatic food and an increasingly important economic activity.

1.2. Effluent management in aquaculture

While aquaculture production continues to increase, the diversity and expansion of this sector also raises potentially problematic issues such as environmental pollution. This is not the case, however, in systems where several species with different trophic levels are incorporated in the same system, and where by-products from one species are recycled to become input for another species, thus reducing wastes from aquaculture into the environment (e.g. the integrated culture of finfish, bivalves and seaweed). To maintain adequate environmental water conditions, a maximum of suspended and dissolved wastes originating from aquaculture should be removed before discharging into the environment or reusing. The elimination of particulate aquaculture waste can efficiently be done in a direct way by filter feeder organisms while dissolved waste, mainly nitrogen, the end product of protein metabolism, can be removed in an indirect way via macroalgae (seaweed) and microscopic organisms (microalgae and bacteria). For removal of dissolved wastes from aquaculture systems, three natural pathways occur (Ebeling et al., 2006): conversion of ammonia-nitrogen via photoautotrophic algae directly into algal biomass, chemoautotrophic bacterial conversion of ammonia-nitrogen to nitrate-nitrogen, and heterotrophic bacterial conversion of ammonia-nitrogen directly to microbial biomass.

Photoautotrophic organisms include terrestrial plants, macroalgae, microalgae and some bacterial species. They can fix carbon dioxide by photosynthesis and utilize excess nutrients (nitrogen and phosphorus) efficiently (Lee and Lee, 2002). The dissolved waste in water is also removed by chemoautotrophic bacteria. These are chemosynthetic organisms, which synthesize organic matter from carbon dioxide and mineral compounds utilizing the energy of certain chemical reactions. There are two phylogenetically distinct groups of bacteria that collectively perform nitrification, in which ammonia is first converted to nitrite (NO_2^-) and then to nitrate (NO_3^-). Ammonia-oxidizing bacteria obtain energy by catabolizing un-ionized ammonia to nitrite (Hagopian and Riley, 1998), and nitrite-oxidizing bacteria carry out the

oxidation of nitrite to nitrate. In both these cases, bacteria obtain energy for growth and maintenance. Heterotrophs finally, in contrast with autotrophs, are organisms which use organic compounds as the source of carbon.

1.3. Promoting heterotrophic bacterial growth in aquaculture systems

A new approach in aquaculture to reduce dissolved waste from the target animals is the biofloc technique which stimulates heterotrophic bacterial growth within the culture system, The bioflocs may be used as food by the target animals, thus resulting in savings on commercial food and on water renewal. Heterotrophic bacteria obtain carbon and energy for growth from naturally occurring organic compounds (McGraw, 2012). Heterotrophic bacterial conversion of ammonia–nitrogen to cell biomass can be enhanced by the addition of organic carbon in the form of sucrose, glucose and other carbohydrate sources (Ebeling et al., 2006). A significant reduction of nitrogen load in aquaculture systems after carbohydrate addition to stimulate heterotrophic bacteria growth has been reported in many studies (Avnimelech, 1999; Burford et al., 2004; Hari et al., 2006). A variety of carbohydrate sources has been used to stimulate bacterial growth in limited or zero water exchange systems, i.e. systems in which the culture water is not renewed during the culture period (Table 1.1).

Table 1.1: Summary of the carbon source and respective species used in limited or zero water exchange culture systems (after Emerenciano et al., 2012)

Carbon source	Culture species	Culture systems	References
Acetate	*Macrobrachium rosenbergii*	5 L lab-scale tanks	Crab et al. (2009a)
Cassava meal	*Penaeus monodon*	1 m^3 lab-scale plastic tanks	Avnimelech and Mokady (1988)
Cellulose	*Oreochromis niloticus*	1 m^3 lab-scale plastic tanks	Avnimelech et al. (1989)
Corn flour	Hybrid tilapia (*O. niloticus* x *Oreochromis aureus*) and hybrid bass (*Morone saxatilis* x *Morone chrysops*)	30 m^3 greenhouse ponds	Milstein et al. (2011)
Dextrose	*Litopenaeus vannamei*	310 L lab-scale plastic tanks	Suita (2009)
Glycerol and glycerol+*Bacillus*	*M. rosenbergii*	5 L lab-scale tanks	Crab et al. (2009a)
Glucose	*P. monodon*	1 m^3 lab-scale plastic tanks	Avnimelech and Mokady (1988)
	M. rosenbergii	5 L lab-scale tanks	Crab et al. (2009a)
Maize starch	*L. vannamei*	18 m^3 outdoor concrete tanks	Liu et al. (2014)
Molasses	*L. vannamei*	40 m^3 greenhouse raceways	Samocha et al. (2007);
		plastic lined 1.8 m-deep ponds	Burford et al. (2004)
	Farfantepenaeus brasiliensis	circular floating cages	Emerenciano et al. (2012)

Table 1.1, continued

Carbon source	Culture species	Culture systems	References
Rice bran	*Artemia franciscana*	120 m^2 earthen ponds	Ronald et al. (2013)
Sorghum meal	*O. niloticus*	1 m^3 plastic tanks	Avnimelech et al. (1989)
Starch	*Oreochromis mossambicus*	1 m^3 tanks	Avnimelech (2007)
	O. niloticus x *O. aureus*	50 m^3 circular concrete ponds	Crab et al. (2009b)
Sucrose	*L. vannamei*	161 m^3 indoor fiberglass tanks	Xu and Pan (2012),
	Marsupenaeus japonicus	185 L tanks	Gao et al. (2012),
		30 m^3 concrete ponds	Zhao et al. (2012),
Sugar	*L. vannamei*	161 m^3 indoor fiberglass tanks	Xu et al. (2012)
Tapioca	*O. niloticus*	704 m^3 circular plastic tanks	Nootong et al. (2011)
	M. rosenbergii	40 m^2 ponds	Asaduzzaman et al. (2008)
	Penaeus monodon	1200 L plastic tanks	Hari et al. (2004)
	A. franciscana	120 m^2 earthen ponds	Sui et al. (2013)
Wheat flour	*O. niloticus*,	250 L fiberglass indoor tanks	Azim and Little (2008)
	F. brasiliensis	circular floating cages	Emerenciano et al. (2012)

Bacteria growing at high densities tend to form bioflocs (Crab et al., 2007; De Schryver and Verstraete, 2009), which are conglomerates of bacteria, protozoa, algae, detritus etc. Bioflocs can be produced by treatment of aquaculture effluents (Kuhn et al., 2010) Bioflocs vary in size from 0.1 mm to a few mm (Avnimelech, 2011). Moreover, the floc structure and floc size is affected by operational parameters such as mixing intensity and shear rate, temperature, and dissolved oxygen (De Schryver et al., 2008).

Burford et al. (2004) reported that more than 29% of the daily food consumed by *L. vannamei* could be bioflocs. The proximate composition of bioflocs is dependent on the carbohydrate source (Crab et al., 2009a). Emerenciano et al. (2012) reported that the protein content of bioflocs grown on acetate and glycerol is higher than in bioflocs grown on glucose, whereas the lipid content shows the inverse relationship. In general, the following ranges of proximate composition of biofloc dry matter have been observed (Crab et al., 2009a; Kuhn et al., 2010): protein is a major component: 19 to 49%; carbohydrates 14 to 59%; ash 3 to 20%; crude lipids 17 to 41%; and crude fiber 13 to 18%.

Bioflocs may thus allow for an additional retention of 7-13% of the proteins supplied by aquafeeds (Hari et al., 2004; Schneider et al., 2005; Hargreaves, 2006). Burford et al. (2004) reported that more than 29% of the daily food consumed by *L. vannamei* could be bioflocs. Promotion of heterotrophic bacterial growth thus not only significantly increases biomass production of target animals, such as filter-feeding organisms, but also reduces artificial feed requirements and improves water quality (Avnimelech, 1999; Burford et al., 2004; Hari et al., 2004, 2006; Nootong et al., 2011).

1.4. Filter-feeding organisms

The term filter feeder relates to an aquatic animal, which takes food by filtering suspended particles from the water, such as particulate waste, microalgae, yeast, bacteria etc. Filter feeders include larvae and adults of some fish species, tunicates, bivalves, sponges, rotifers and various zooplanktonic crustaceans such as copepods and *Artemia*. Zooplankton species are mostly an important feed source for fish and shellfish larvae. Characterized by the possession of an organ used to capture suspended particles from the water, filter feeders show a highly variable array of feeding structures such as appendages bearing setae, mucus or silk nets, gill rakers

and baleen plates, lophophores, tentacles, and ciliated and flagellated cells (Humphries, 2009).

Suspension-filtering fishes, both selective and non-selective filter feeders, consume vast numbers of tiny prey by passing large quantities of water through their oropharyngeal cavity (Sanderson et al., 1994; Cheer et al., 2001). Food particles may then be trapped on gill rakers and transported to the esophagus. The size of filtered particles is dependent on the stage and species of fish.

The majority of bivalve mollusks are filter feeders, both larvae and many adult bivalves suspension-feed by capturing microscopic particles, including detritus, bacteria, microalgae and small animals, with particles typically having diameters in the order of 1 to 100 μm (Dame et al., 1993). The feeding mechanism of bivalves is a process of active sorting of particles based on particle size (Defossez and Hawkins, 1997; Lehane and Davenport, 2006), shape (Bougrier et al., 1997), nutritive value (MacDonald and Ward, 1994; Hawkins et al., 1996; 1998) or chemical component on the surface of the particle (Yahel et al., 2009). The food particles of adequate size are removed from the water by filtration and are then sorted by the palps on the labia into ingestible particles and non-ingestible particles; the latter are encased in mucus and are then rejected from the mouthparts as pseudofaeces (Beecham, 2008).

Rotifers are zooplankton organisms filter-feeding on microscopic particles. Small-size rotifers, such as *Brachionus angularis*, *Filinia longiseta* and *Pompholyx sulcata,* filter bacteria-size particles as efficiently or more efficiently than larger particles (Ooms-Wilms, 1997).

Larval crustaceans (cladocerans, copepods, *Artemia* and other anostracans) have setae on the second appendages (second antennae) that act as filters and the food is taken up by the mandibles. The filtering process in adult crustaceans has been described by Geller and Müller (1981) as occurring in three steps: (1) capturing of particles during swimming activities by setae or bristles of lobed thoracic legs at first contact; (2) removing of captured particles from the filter-combs of lobed thoracic legs by means of long bristles acting as “sweeper” and collecting them into the mid-ventral food groove; (3) transport of food along the food groove towards the mouth opening. The distance between setae of *Daphnia* species increases from 0.3-0.4 μm in juveniles to 0.8-2.0 μm in adults (Geller and Müller, 1981). *Daphnia* usually

consumes particles from 1 to 50 µm, although particles of up to 70 µm may be found in the gut of large individuals (Ebert, 2005). Similarly, *Artemia*, a non-selective filter feeder, can consume all particles in the water column with a size less than 50 µm in case of adults (D'Agostino, 1980), while the food size for *Artemia* metanauplii must range between 6.8 to 27.5 µm, with an optimum of about 16.0 µm (Fernández, 2001). In addition, Makridis and Vadstein (1999) reported that the inter−setular distance in antennae of 2−day−old *Artemia* metanauplii was 0.20 ± 0.07 µm, and that the inter-setular distance in thoracopods of 4− and 7−day−old *Artemia* metanauplii was 0.16 ± 0.05 and 0.18 ± 0.04 µm, respectively, which allows them to graze on bacteria−size particles (0.5 µm).

1.5. Food types and food consumption in filter feeders

Thanks to their filter-feeding characteristics, filter feeders are able to graze on a variety of microscopic cells suspended in the water column, including microalgae, yeast, bacteria etc.

1.5.1. Microalgae

Microalgae are unicellular organisms capable to convert solar energy into chemical energy via photosynthesis (Hosikian et al., 2010; Priyadarshani and Rath, 2012). They vary in size from few µm to 2 mm and occur in most aquatic habitats (Griffiths et al., 2011). Microalgae can produce biomass very rapidly, with some species doubling in as few as 6 h, and many exhibiting two doublings per day (Huesemann et al., 2009; Hannon et al., 2010). The total content of lipids in microalgae may vary from 1 to 80% of their dry weight (Chisti, 2008; Satyanarayana et al., 2011), with protein contents in the range 10−60% and carbohydrates 7−40% (Satyanarayana et al., 2011), in addition to other nutritional compounds, such as vitamins, pigments, minerals and other trace elements.

Microalgae are the basis of the aquatic food chain and are an essential nutrition source for filter feeders, including live food and the earliest stages of aquatic species in hatcheries. Microalgae are often the standard diet used for *Artemia* laboratory culture (Table 1.2). Among the variety of microalgae species used, *Tetraselmis suecica* has been repeatedly reported as one of the best species for ongrowing of *Artemia* (Seixas et al., 2009).

Table 1.2: Microalgal species used in *Artemia* laboratory culture (Seixas et al., 2009).

Authors	Microalgae species used to feed *Artemia* sp.	Temperature of rearing	Best microalgal diet	Length of *Artemia* sp.
Sick (1976)	Five species tested: *Chlamydomonas sphagnicola*, *Dunaliella viridis*, *Chlorella conductrix*, *Platymonas elliptica*, *Nitzschia closterium*	25 °C	*C. sphagnicola* *D. viridis*	1.8 mm (day 6) 8.9 mm (day 16) 2.9 mm (day 6) 5.7 mm (day 16)
Abreu-Grobois et al. (1991)	*Dunaliella tertiolecta*	28 °C		10.0 mm (day 14)
Gamallo (1992)	Six species: *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Dunaliella tertiolecta*, *Isochrysis galbana*, *Nannochloris atomus*, *Nitzschia acicularis*	25 °C	*T. suecica* *D. tertiolecta*	4.6 mm (day 8) 4.4 mm (day 8)
Fábregas et al. (1996)	*Tetraselmis suecica*	25 °C		8.3 mm (day 19)
Evjemo and Olsen (1999)	*Isochrysis galbana* T-ISO	26–28 °C		3.3 mm (day 6) 5.9 mm (day 12)

Table 1.2, continued

Authors	Microalgae species used to feed *Artemia* sp.	Temperature of rearing	Best microalgal diet	Length of *Artemia* sp.
García-Ulloa Gómez et al. (1999)	*Tetraselmis suecica* *Chaetoceros calcitrans* *Spirulina* sp. (dried)	25.5 °C	*T. suecica*	2.1 mm (day 6) 4.9 mm (day 10)
			Spirulina sp. (dried)	3.2 mm (day 6) 4.7 mm (day 10)
Naegel (1999)	*Chaetoceros* sp.	25 °C		2.0 mm (day 6) 4.6 mm (day 11)
Thinh et al. (1999)	Thirteen species of tropical Autralian microalgae (benthic and planktonic)	25 °C	*Cryptomonas* sp.	0.92 mm (day 1)
			Chaetoceros sp.	0.89 mm (day 1)
			Tetraselmis sp.	0.88 mm (day 1)
			Cryptomonas sp.	6.5 mm (day 7)
			Chaetoceros sp.	5.5 mm (day 7)
Godínez et al. (2004)	*Tetraselmis suecica* *Chaetoceros muelleri*	25 °C	*T. suecica*	4.5 mm (day 10)
			C. muelleri	3.7 mm (day 10)
Lora-Vilchis et al. (2004)	*Chaetoceros muelleri* *Isochrysis galbana* T-ISO	27.5 °C	*C. muelleri*	6.0 mm (day 7)
			I. galbana T-ISO	4.2 mm (day 7)

Table 1.2, continued

Authors	Microalgae species used to feed *Artemia* sp.	Temperature of rearing	Best microalgal diet	Length of *Artemia* sp.
Marques et al. (2004a)	*Dunaliella tertiolecta* (two strains: 19/6B; 19/27) *Tetraselmis suecica* (two strains: 66/4; 66/22A)	28 °C	*T. suecica* 66/4 *T. suecica* 66/22A *D. tertiolecta* 19/6B	3.5 mm (day 6) 4.0 mm (day 6) 3.2 mm (day 6)
Seixas et al. (2009)	*Tetraselmis suecica*, *Rhodomonas lens*, *Nannochloropis gaditana*, *Isochrysis galbana*	26.5 °C	*R. lens*	3.6 mm (day 5) 4.9 mm (day 8)

1.5.2. Yeast

Another food source for filter-feeding zooplankton is yeast. Yeasts are aquatic and terrestrial unicellular fungi with good buoyancy in the water column. The cell size of yeasts varies according to the species, but in general ranges from 2.5 to 10.5 µm in width and 4.5 to 21.0 µm in length (Reed and Peppler, 1973). According to Waslien and Oswald (1975) yeasts are a rich source of proteins (45% of yeast cell dry weight), and contain 4−7% lipids, 26−36% carbohydrates and 5−10% ash. Being protein-rich, yeasts are attractive food supplements or substitutes in aquaculture and their production costs are lower as compared with microalgae (Coutteau et al., 1992). Particularly baker´s yeast *Saccharomyces cerevisiae* has been used to culture live food such as rotifers (Hirayama and Funamoto, 1985) and *Artemia* (Coutteau et al., 1990; Marques et al., 2004a; Soltanian et al., 2007).

1.5.3. Bacteria

Bacteria are grazed by filter feeders as well and can make up for a substantial part of their diet. They satisfy between 26.7% and 70.6% of metabolic demand for nitrogen in the Japanese oyster species *Crassostrea gigas* and ribbed mussel *Geukensia demissa* during the spring period when metabolic activity is high (Langdon and Newell, 1990). Moreover, the contribution of bacteria in the diet of the mussel *Mytilus galloprovincialis* has been found to be of constantly moderate level in all seasons (Prato et al., 2010). The ingestion of suspended bacteria has been demonstrated in fry of tilapia (*Oreochromis niloticus*) (Beveridge et al., 1989), fingerlings of common carp (*Cyprinus carpio* L.) (Beveridge et al., 1991), and fry of other carp species (Rahmatullah and Beveridge, 1993). Furthermore, in recent years the ingestion of bacteria by filter feeders in general has been demonstrated by using the fluorescent-labeling technique to tag bacterial cells (Soto-Rodriguez et al., 2003; Niu et al., 2012; Hisatugo et al., 2013).

For some filter-feeding zooplankton species, bacterial cell size has been demonstrated as suitable for filtering in all life stages. Measuring grazing by using fluorescent particles of 0.5, 2.4 and 6.3 µm, Ooms-Wilms (1997) reported that *Anuraeopsis fissa*, a small rotifer, filtered bacteria-sized particles more efficiently than the larger particles. In contrast, three other rotifer species (*Brachionus angularis*, *Filinia longiseta* and *Pompholyx sulcata*) filtered the bacteria-sized

particles less efficiently than the larger particles. In a study using carbon and nitrogen stable isotope analysis of zooplankton and several of their putative food sources, Taipale et al. (2008) found that among four potential main food sources (phytoplankton, heterotrophic bacteria, methanotrophic bacteria and green sulfur bacteria), the dietary contribution of heterotrophic bacteria to *Daphnia* ranged from 50 to 58% during summer.

The major component of bacterial cells is proteins (25−49% of dry weight), followed by ash (4.7−14.0%) and lipids (2.5−9.0%), illustrating the potential of bacteria as source of nutrients (Brown et al., 1996). However, bacteria lack poly-unsaturated fatty acids (PUFA) and sterols, which are essential nutrients for the growth of marine organisms (Phillips, 1984). In the natural aquatic environment, filter-feeding organisms can get plenty of food sources, and the PUFA and sterol deficiency in bacteria can be compensated from orher food sources (e.g. microalgae). Moreover, when PUFA are not obtained from the diet, they can be bioconverted by certain organisms from precursor FA. The bioconversion from short-chain fatty acids into long-chain poly-unsaturated fatty acids has been demonstrated in the harpacticoid copepod *Microarthridion littorale*, where unidentified bacteria, originated from pond sediment, were used as monodiet (De Troch et al., 2012).

As for *Artemia*, initial efforts to culture brine shrimp on a diet using solely bacteria failed (Seki, 1964; D'Agostino, 1980). In contrast, several later studies have demonstrated that *Artemia* survives well on bacteria and that bacterial food results in good *Artemia* growth (Table 1.3).

Table 1.3: Overview of the use of bacteria as a food source for *Artemia* nauplius

Author	Bacteria strains	Culture method	Stocking density	*Artemia* length/survival
Yasuda and Taga (1980)	*Acinetobacter* sp. strain B-9	Xenic	5 nauplii/mL	3.5 mm (12 days)
Intriago and Jones (1993)	*Flexibacter* strain Inp3	Xenic	1 nauplius/mL	4.5 mm (8 days)
Rico-Mora and Voltolina (1995)	SK-01 (*Vibrio* sp.)	Gnotobiotic	1 nauplius/mL	~ 40% (4 days)
	SK-02 (*Vibrio* sp.*)*			~ 60% (4 days)
	SK-03 (*Flavobacterium*)			~ 70% (4 days)
	SK-04 (*Plesiomonas*)			~ 75% (4 days)
	SK-05 (*Aeromonas*)			~ 20% (4 days)
Verschuere et al. (1999)	LVS4 (*Moraxella*)	Gnotobiotic	1 nauplius/1.5 mL	~ 2.3 mm (6 days)
	LVS5 (*Alcaligenes*)			~ 1.5 mm (6 days)
Marques et al. (2005)	LVS2 (*Bacillus* sp.)	Gnotobiotic	1 nauplius/1.5 mL	1.2 mm (6 days)
	LVS3 (*Aeromona hydrophila*)			1.6 mm (6 days)

1.6. Beneficial effects of bacterial supplements in filter feeding zooplankton

The growth rate of rotifer (*Brachionus plicatilis*), obtained when fed baker's yeast (*Saccharomyces cerevisiae*) with the individual or joint addition of *Lactococcus casei* ssp. *casei, Pediococcus acidilactici,* or *Lactobacillus lactis* ssp. *lactis,* was 8–13 times higher than in cultures without bacteria addition (Planas et al., 2004). Moreover, the growth rate of rotifers significantly improved when *Alteromonas* bacteria were added one time at the first day of the culture period as compared to solely microalgae-fed rotifers (Douillet, 2000). Similarly, the improvement of growth and survival of *Artemia* by bacteria addition has been demonstrated by Douillet (1987) in an experiment, where unidentified bacteria previously selected from xenic cultures sustaining better *Artemia* performance were added to an *Artemia* culture, as compared to solely inert diets-fed *Artemia*.

The increased growth rate of consumers in case of bacteria addition is generally linked to digestive enzyme activities (Ziaei-Nejad et al., 2006). Increase of digestive enzymes may lead to enhanced digestion and absorption of food, which in turn contributes to the improved survival and growth of consumers. Bacteria are considered a source of exogenous extracellular enzymes (Verschuere et al., 1999; Maki et al., 2009). The increase of enzyme activities with the addition of specific bacterial strains in the culture medium has been demonstrated in non-filter feeding crustaceans such as marine larval shrimp *Litopenaeus vannamei* and *Penaeus* sp. (Rengpipat et al., 1998; Zhou et al., 2009; Nimrat et al., 2012), and on filter-feeding species such as rotifer *Brachionus plicatilis* (Douillet, 2000a, b; Planas et al., 2004), and *Artemia* (Douillet, 1987; Intriago and Jones, 1993; Gorospe and Nakamura, 1996).

1.7. Use of biochemical tracers in nutritional studies

The criteria growth rate and survival are commonly used as indirect indicators to evaluate dietary performance in consumers. In recent years, biochemical tracers, such as isotopes and fatty acids, have been applied to study the assimilation of nutrients from diets by the consumer (Pitt et al., 2009). Stable isotopes (e.g. ^{13}C, ^{15}N, ^{34}S) or unstable (radioactive) isotopes (e.g. ^{14}C, ^{3}H) are broadly used to estimate the energy flow in trophic relationships, because different food items have different isotope signatures. Delta values (δ) in per mil (‰) are used to express the relative

difference between the isotope ratio of the sample and that of a standard with known isotopic composition (Fry, 2006; Coplen, 2011).

$$\delta\ (‰) = \frac{R_{Sample} - R_{Standard}}{R_{standard}} \text{ x } 1000$$

where R is ratio of the concentrations (atom fraction) of the heavy and light isotopes (e.g. $^{15}N/^{14}N$ isotope concentration ratios). $R_{standard}$ is the ratio of an internationally recognized standard (e.g. the standard for N is atmospheric N_2).

The terms trophic shift or fractionation factor, previously used to describe the isotopic difference between the consumer and its diet (Cerling et al., 2007), have been replaced by the term isotope discrimination factor in recent years. The carbon and nitrogen isotopic signatures of animals typically reflect the isotopic signatures of their diets plus a discrimination factor (isotopic discrimination, $\Delta = \delta_{muscle\ tissue} - \delta_{diet}$) caused by the different isotopes of the same element being incorporated into muscle tissues of the consumer at different rates (Peterson and Fry, 1987; Le Vay and Gamboa-Delgado, 2011). Apart from using natural isotope abundance of the prey, stable isotopes are increasingly being used as tracer in nutritional studies to follow the fate of specific dietary components. Labeling of live diets has been typically used to quantify larval feed intake and/or to characterize digestion, absorption, metabolism or retention of dietary nutrients (Conceição et al., 2007). Stable isotopes have for example been used for nitrogen budget studies of marine zooplankton species (Hino et al., 1997) and to study the grazing efficiency of copepods (De Troch et al., 2007). Also the ingestion and assimilation of bacteria by *Artemia* has been reported using unstable isotope ^{14}C as a tracer (Fernández, 2001).

Fatty acids (FA) profile analysis is another tool for dietary investigations because the FA composition of the diet is reflected in the liver and the muscle tissue of the consumers (Beckmann et al., 2012). The assimilation of bacteria by rotifers has been demonstrated when feeding rotifers with eicosapentaenoic acids (EPA)–producing bacteria; the level of EPA in rotifers reached 9.4% of total fatty acids after 24 h of feeding, while this fatty acid in unfed rotifers was as low as 1.0% (Nichols et al., 1996). Similarly, FA biomarkers have been applied to illustrate the assimilation of bacteria by *Artemia*: 16:1n-7 and 18:1n-7, major fatty acids present in bacteria, showed also higher levels in bacteria-fed *Artemia* compared with non-bacteria fed animals (Intriago and Jones, 1993).

1.8. *Artemia*

1.8.1. Biology and ecology of *Artemia*

The brine shrimp *Artemia* is a small crustacean zooplankton species and an important live food in larviculture. Due to their nutritional value and appropriate 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). *Artemia* lives in hypersaline biotopes throughout the world in which the salt content may be up to 250 g/L (Sorgeloos, 1980). The systematic classification of the genus is as following:

Phylum: Arthropoda

Class: Crustacea

Subclass: Branchiopoda

Order: Anostraca

Family: Artemiidae

Genus: *Artemia*, Leach 1819

The genus *Artemia* contains sexual species and parthenogenetic lineages. *Artemia* can reproduce in two ways (Fig. 1.2). If living conditions are favorable, the fertilized eggs in the brood pouch of the female develop into free-swimming *Artemia* nauplii (ovoviviparous reproduction). If living conditions deteriorate, *Artemia* has the ability to produce dormant embryos about 200-300 µm in size, known as cysts, that are in a state of obligate dormancy called diapause (Lavens and Sorgeloos, 1987).

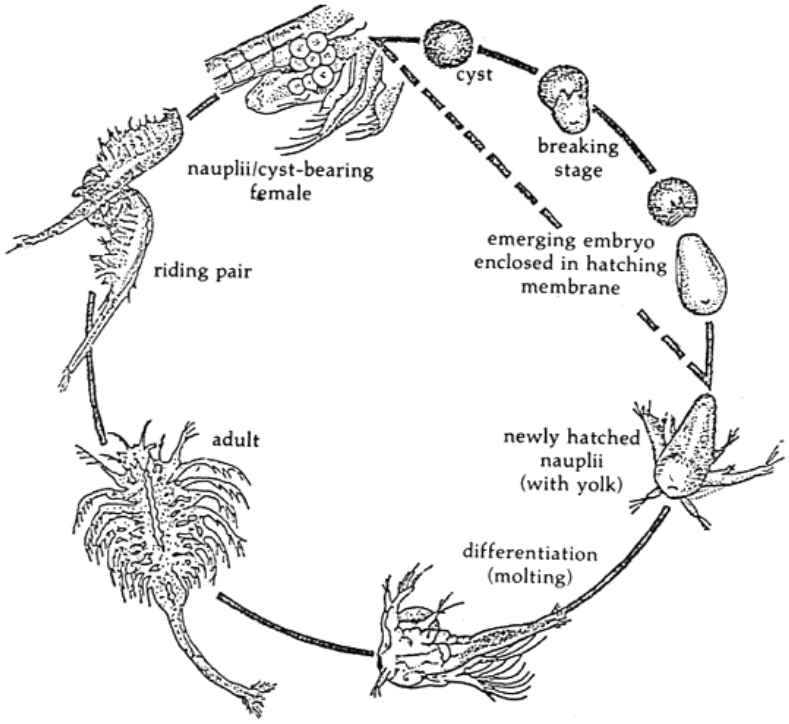

Figure 1.2: Life cycle of *Artemia* (Jumalon et al., 1981)

In highly saline water conditions, cysts float on the water surface and are blown to the shore by wind and wave action, where they accumulate. The cysts are naturally dried by salt and sun. The cysts resume metabolism and further develop when being hydrated, after being exposed to external diapause breaking conditions. During hydration, the aerobic metabolism in the cysts assures the conversion of the carbohydrate reserve trehalose into glycogen (as a source of energy) and glycerol, resulting in further water uptake by the embryo (Van Stappen, 1996). Increased levels of the latter hygroscopic compound thus lead to hyperosmotic pressure. The cyst shell bursts (after approximately 8−20 h) and the embryo with surrounded hatching membrane ("umbrella") appears (Van Stappen, 1996). After breaking from the enclosing membrane, the free swimming *Artemia* nauplius (instar I) can be observed. The early larval stage with a length of about 400−500 µm does not feed, but relies on its energy store in the yolk, which gives this stage of *Artemia* a brownish-orange color. The nauplius has a red nauplius eye in the head region and three pairs of appendages (Fig. 1.3 A) (Van Stappen, 1996): the first pair of appendages (first antennae) has a sensorial function, the second appendages (second antennae) have locomotory and filter-feeding functions, and the mandibles have a food uptake function. The ventral side is covered by a large labrum (for food

uptake through transfer of particles from the filtering setae into the mouth). After a few hours, the larva molts into the instar II stage.

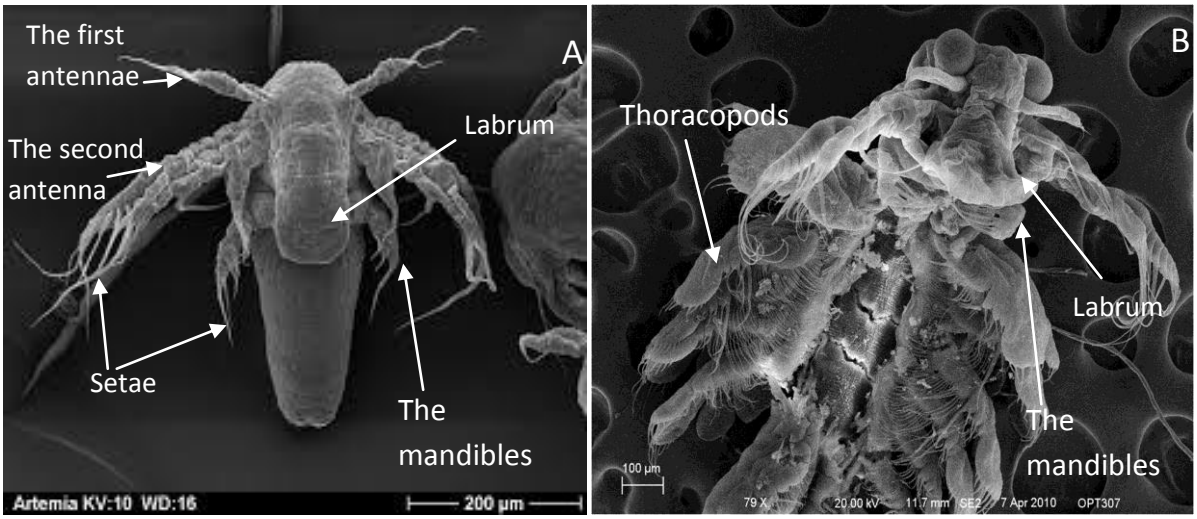

Figure 1.3: *Artemia* nauplius (A) with three pairs of appendages, and the adult *Artemia* (B) with 11 pairs of thoracopods

(source: http://www.optics.rochester.edu/workgroups/cml/opt307/spr10/jonathan/)

The larva undergoes 15 molts in about 2 weeks until the adult stage depending on the culture conditions (Clegg and Conte, 1980; Sorgeloos, 1980). Paired lobular appendages appear in the trunk region and differentiate into thoracopods. From the 10^{th} instar stage on, important morphological as well as functional changes are taking place (Van Stappen, (1996): i.e. the antennae lose their locomotory function and undergo sexual differentiation. The thoracopods differentiate into three functional parts (Fig. 1.3 B and Fig. 1.4) namely the telopodites and endopodites (locomotory and filter-feeding), and the membranous exopodites (gills). The adult has a body length of 8−10 mm with two lateral eyes, a linear digestive tract and 11 pairs of thoracopods. The adult female *Artemia* is recognized by the presence of the brood pouch or uterus between cephalothorax and abdomen. The adult male possesses a paired penis in the posterior part of the trunk and muscular graspers in the head region.

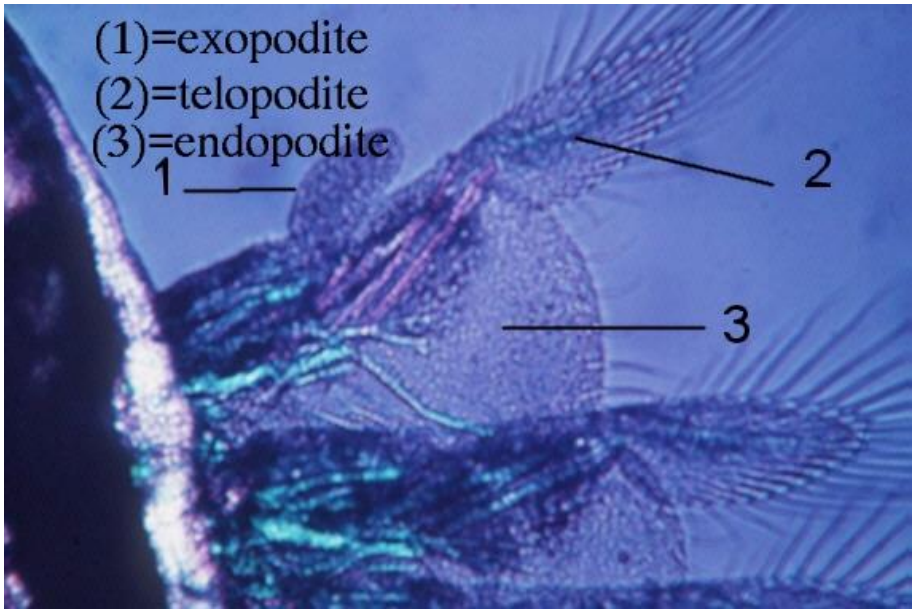

Figure 1.4: Detail of anterior thoracopods in adult *Artemia* (Van Stappen, 1996)

1.8.2. Role of *Artemia* in aquaculture

Annually, 2500-3000 tonnes of dry *Artemia* cysts, mainly harvested in inland salt lakes such as Great Salt Lake, Utah, USA, are used in aquaculture hatcheries worldwide (FAO, 2011). *Artemia* cysts are stored easily over long periods and live nauplii are obtained within 24 h by incubating cysts in seawater. Moreover, *Artemia* is used as a vector to transfer bioencapsulated nutrients or pigments to target animals thanks to their non-selective filter feeding characteristics (Sorgeloos et al., 2001). Due to these convenient characteristics, the demand of *Artemia* cysts for aquaculture hatcheries has continuously increased over the past few decades.

Also on-grown *Artemia* biomass is used in aquaculture. According to Anh et al. (2009a), proteins and lipids are the major components in on−grown *Artemia*, which can be used to replace *Artemia* nauplii in specific aquaculture practices such as feeding post-larval *Penaeus monodon* (Dhert et al., 1993) and larvae of mud−crab (*Scylla serrata*; *Scylla paramamosain*) (Mann et al., 2001; Anh et al., 2011). Moreover, *Artemia* protein has been used to replace fish meal for postlarval fresh-water prawn (*Macrobachium rosenbergii*) (Anh et al., 2009a).

1.8.3. *Artemia* culture

The production of *Artemia* cysts from inland salt lakes is not constant and the demand of this product for hatcheries sometimes exceeds the supply. Moreover, there is also a demand for a variety of cyst products with different qualitative

characteristics (Bengtson et al., 1991; Sorgeloos et al., 2001). Trying to meet these demands, the technique of *Artemia* culture in salt ponds has been introduced throughout the world, such as in Brazil in 1977 (Camara and Tackaert, 1994), the Philippines in 1977 (De Los Santos et al., 1980), Thailand (Vos and Tunsutapanich, 1979) and Vietnam in the 1980´s (Brands, 1996), Pakistan in 2007 (Sultana et al., 2011), India during the 1980´s (Sivagnanam et al., 2011) and Kenya in 2012 (unpublished data).

Among the countries which have introduced *Artemia* culture into the salt production ponds, as mentioned above, *Artemia* culture in Vinh Chau and Bac Lieu provinces, Vietnam, is considered as particularly successful. An additional source of income can be generated by integrated *Artemia* cysts and salt production, thus helping poor salt famers to improve their living standards.

Artemia does not occur naturally in this area; because of the monsoon climate, natural highly saline water, where *Artemia* might occur, is not available. Highly saline water is prepared in the culture systems by evaporation during the dry season, which lasts from November to May in southern Vietnam. In the traditional culture method and management, *Artemia* instar I nauplii are inoculated at 100-150 nauplii/L into ponds with a water salinity ranging from 80 to 120 g/L where predators (e.g. tilapia) have been eliminated (Hoa, 2014). Being a filter feeder, *Artemia* can be reared in the field with microalgae, which are stimulated to grow by organic and inorganic fertilizers in separate fertilizer ponds having salinity around 35 g/L. Moreover, soybean powder, rice bran, chicken manure and pig dung (Anh, 2009) etc. can be used as supplemented feed. Raking of the pond bottom is regularly applied to re-suspend the particles accumulated on the pond bottom and to prevent the growth of benthic macroalgae. In these conditions, *Artemia* generally starts to reproduce within two or three weeks (Hoa, 2014).

In current years, the cyst production in this area (Fig. 1.5) is mainly used for local hatcheries.

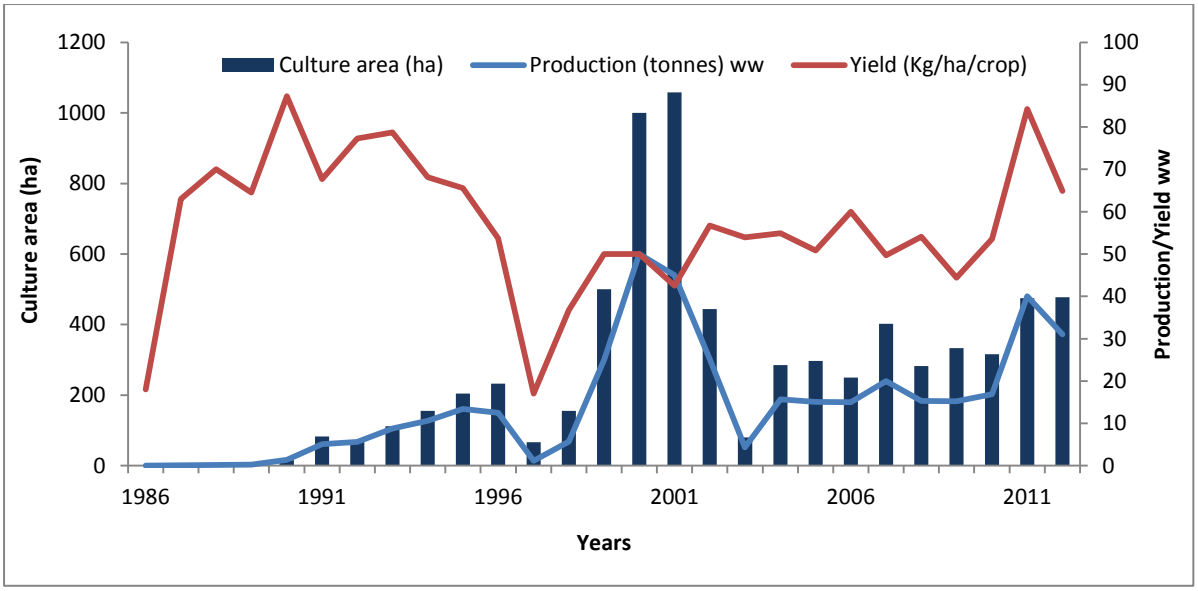

Figure 1.5: Culture area, total production and yield of *Artemia* cysts (wet weight product) in the Vinh Chau and Bac Lieu districts of the Mekong Delta, Vietnam, (Source: College of Aquaculture & Fisheries, Can Tho University, Vietnam).

1.9. Rationale and objectives of the study

In the management typical of *Artemia* pond culture in Vietnam as described above, phytoplankton, which is stimulated to grow by inorganic and organic fertilizers, is used as a main food source and rice bran is mostly used as supplemented feed for *Artemia* at 40-60 kg/ha (Baert et al., 1997; Anh et al., 2009b). There is, however, no information available about the contribution of bacteria, naturally occurring in the culture systems, to the *Artemia* diet. Moreover, *Artemia* is mostly reared in stagnant pond systems, and excess food and waste from *Artemia* accumulate in the pond during the culture period. If the waste from *Artemia* culture is not well managed, it can reduce survival and growth of *Artemia* and affect the environment. In recent years, aquaculture effluents are sometimes managed by adding carbohydrates to stimulate heterotrophic bacterial growth, which in turn can be used as a natural food source for some target consumers. Promoting bacterial growth in aquaculture systems clearly adds to environmental quality (Hari et al., 2006) and reduces the demand of artificial feed (Avnimelech, 1999; Hari et al., 2004). This so-called biofloc technique has been applied in pond based *Artemia* culture systems too (Ronald et al., 2013; Sui et al., 2013). However, external field factors may interfere with the set-up (Ronald et al., 2013): in open *Artemia* culture systems uncontrollable variations in many parameters may occur, making it extremely difficult to evaluate the contribution

of a single diet (bacteria) among a wide variety of foods (algae, bacteria, detritus particles…) available at the same time. Consequently the results obtained from these field studies did not allow clear conclusions about the contribution of heterotrophic bacteria to the *Artemia* diet. Therefore the main objective of our study was to determine to what extent *Artemia* may take nutritional advantage from associated bacteria. By working within controlled laboratory conditions, we aimed to find out if bacteria may be a substitute dietary source for other types of food in *Artemia*, assuming that this information may contribute to our understanding of *Artemia* feeding processes in pond conditions.

For this purpose, firstly heterotrophic bacteria were separated from other food sources and fed to germ-free *Artemia* as mono-diet under laboratory culture conditions; these nine bacterial strains were isolated from the culture medium of a well-performing *Artemia* culture. *Artemia* nauplii were offered the bacterial isolates in a 6-day experimental period under gnotobiotic culture conditions, as described in **Chapter 2.** The survival and growth of the *Artemia* fed the respective heterotrophic strains were measured, and those bacterial strains supporting adequate *Artemia* growth and survival were withheld for further research.

To sustain good animal growth, nitrogen is quantitatively the most important nutrient. It is a major constituent of essential compounds such as proteins. In a subsequent study, the effect of different food sources (heterotrophic bacteria, microalgae and yeast) on the growth of *Artemia* was evaluated by studying N assimilation. In the first set of experiments, heterotrophic bacteria, selected in the previous study based on their positive effect in the gnotobiotic culture of *Artemia* nauplii, were offered to germ-free *Artemia* nauplii together with two strains of the microalga *Dunaliella tertiolecta*, one being a good food for *Artemia*, the other one a suboptimal food source, as proven in literature (Marques et al., 2004a). The N assimilation in *Artemia* from microalgae in the presence of bacteria and vice versa was measured by using the ^{15}N stable isotope tracer as a study tool in comparison with monodiets consisting solely of bacteria or microalgae (**Chapter 2)**.

Analogously, the same heterotrophic bacteria strains as described above were also offered to germ-free *Artemia* nauplii together with two strains of baker´s yeast *Saccharomyces cerevisiae* with different digestibility. The N assimilation from the food in *Artemia* was measured by using the ^{15}N stable isotope tracer as the study

tool. The results of N assimilation in *Artemia* tissue from yeast in the presence of bacteria and the N assimilation from bacteria in the presence of yeast are presented and discussed in **Chapter 3**.

In the set of the above experiments, the role of heterotrophic bacteria in *Artemia* diets was investigated under gnotobiotic experimental conditions. The data obtained from these experiments illustrated to what extent heterotrophic bacteria can be a direct food source or improve N assimilation from other food sources in *Artemia*. In a subsequent study (**Chapter 4**), the biofloc stimulation technique was applied in an *Artemia* culture to determine whether *Artemia* takes advantage from bioflocs when lacking algae supply. Two different carbohydrate sources (sucrose and soluble potato starch) and inorganic nitrogen were added to obtain two different C/N ratios (10 and 50) in the cultures. It was necessary to perform the studies under xenic conditions to allow biofloc growth. To quantify the biofloc consumption in *Artemia*, ^{15}N stable isotope labeled bioflocs were used. The contribution of the bacteria to the *Artemia* diet was evaluated by measuring *Artemia* performance in terms of survival, individual length and total biomass production together with the ^{15}N accumulation and the fatty acids signatures of the *Artemia* tissue at the end of the rearing period.

The carbohydrate source and C/N ratio producing the best results in Chapter 4 were used to stimulate biofloc production in the following experiment in which we aimed to verify from which level of reduction of algal supply onwards *Artemia* takes advantage of the bacteria supply. Fatty acids and ^{15}N stable isotope accumulation in *Artemia* tissue were also measured at the end of the rearing period to quantify the consumption of bioflocs by *Artemia*. These results and *Artemia* performance in terms of survival, individual length and total biomass production are presented in **Chapter 5.**

In Chapter 6, the results produced in the various chapters are discussed. The main conclusions of the overall work are given.

Chapter 2

Co-feeding of microalgae and bacteria may result in increased N assimilation in *Artemia* as compared to mono-diets, as demonstrated by a ^{15}N isotope uptake laboratory study

Modified from:

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Chapter 2: Co-feeding of microalgae and bacteria may result in increased N assimilation in *Artemia* as compared to mono-diets, as demonstrated by a ^{15}N isotope uptake laboratory study

Abstract

This study investigated the contribution of associated bacteria to *Artemia* nutrition. Nine bacterial strains, coded HT1 to HT9, were isolated from a previous *Artemia* culture. These bacteria were daily offered to germ-free *Artemia* nauplii during 6 days of experimental culture period. The survival and length of HT3- and HT6-fed *Artemia* were the best among nine isolates, and comparable with results obtained with probiotic bacteria LVS3 (*Aeromonas hydrophila)*. Subsequently, these bacterial strains were fed in combination with microalga *Dunaliella tertiolecta* strains, either the good quality DT 19/6B, supporting good *Artemia* survival and growth, or the low quality DT 19/27 strain. Each combination of algae and bacteria was offered in different proportions, i.e. 10/90, 50/50 and 90/10% on a dry weight basis, while the total amount of food supplied, which was based on a reference algae mono-diet, was kept constant. Mono-diets consisting of 100% algae and 100% bacteria were added as controls. N assimilation from either food source was determined by feeding in separate tests ^{15}N labeled microalgae with non-labeled bacteria and vice versa. Axenically hatched *Artemia* nauplii were fed these diets for 24 h, after which they were analyzed for ^{15}N content. The results of ^{15}N analysis showed that the N assimilation from bacteria in *Artemia* was improved when the bacteria were 10% replaced by microalgae, and for the combination of DT 19/6B and HT6 this increase was significant ($p < 0.05$). Also 50% replacement of HT6 by DT 19/6B resulted in higher, but non-significant, N assimilation from the bacteria as compared to the 100% bacteria mono-diet. A similar non-significant increase of N assimilation from DT 19/6B was observed as compared to the 100% algae diet when these algae were up to 50% replaced by HT6 bacteria. Other combinations of bacterial and microalgal strains generally produced similar or lower N assimilation as compared to the labeled mono-diet, when the labeled food component (bacteria or algae) was gradually replaced by the other (algae or bacteria). This study shows that, provided suitable strains are used, offering microalgae and bacteria together as food for

Artemia may result in a beneficial effect in how this filter-feeding organism takes advantage of the respective food sources. This *Artemia* study can also be considered as a model for future research on the feeding biology of other filter-feeding aquaculture organisms.

2.1. Introduction

Being among the best sources of live food for cultured fish and shellfish species around the globe, the brine shrimp *Artemia* is demanded extensively by the ever growing world aquaculture industry (Sorgeloos et al., 2001). However, to meet this increasing demand and to diversify the *Artemia* resources, *Artemia* has been introduced into coastal solar saltworks in countries such as Vietnam, Thailand and the Philippines where there is no natural occurrence of *Artemia*.

In the typical pond production system of *Artemia* in saltworks in the Mekong delta, south Vietnam, *Artemia* is fed with algae-rich water from fertilized ponds as a main food source, alternatively supplemented with inert feeds (such as rice bran and soybean meal) and animal wastes (chicken manure, pig dung) (Baert et al., 1997; Anh et al., 2009b). In such open systems, the naturally occurring bacterial flora growing on the non-consumed and decomposing nutritionally rich food can also be a direct source of food for *Artemia*, considering the abundant availability of bacteria and the ability of *Artemia* to ingest them by filter feeding. Though bacteria are believed to be an important food source for filter feeding *Artemia*, the exact dietary role of bacteria in open culture systems, or whether they can be effectively utilized by *Artemia* is poorly understood.

In laboratory conditions, however, the nutritional role of bacteria has been demonstrated by various studies (Yasuda and Taga, 1980; Intriago and Jones, 1993; Gorospe and Nakamura, 1996; Gorospe et al., 1996; Verschuere et al., 1999). The ability of *Artemia* to survive on bacteria alone has been shown previously (Yasuda and Taga, 1980; Intriago and Jones, 1993; Gorospe and Nakamura, 1996; Verschuere et al., 2000), and similarly when using bacteria combined with algae (Intriago and Jones, 1993) or dry inert feeds (Douillet, 1987). Bacteria are considered to be a good source of essential nutrients such as vitamins, fatty acids and amino acids (Austin, 1988; Yu et al., 1988; 1989; Gorospe and Nakamura, 1996; Verschuere et al., 2000). Moreover, bacterial enzymes are believed to play a

prominent role in breaking down large food particles and make them easy to absorb by *Artemia.* Such enzyme degradation using bacterial enzymes has been demonstrated for example for digestion of algae cells by *Artemia* (Intriago and Jones, 1993). The presence of bacteria in the culture medium has been proven to improve growth performance of aquatic animals in general (Intriago and Jones, 1993; Marques et al., 2004a). Associated bacteria are believed to remove toxic metabolites in aquaculture production systems, act as a direct source of nutrients and/or as a promoter of food assimilation. Additionally, beneficial probiotic strains protect the host against infection from pathogenic bacteria (Austin, 1988).

However, literature data do not provide reliable information on the effect of the bacteria, associated with *Artemia* laboratory or pond culture, on food assimilation in *Artemia*.

To optimize the biomass and cyst production in pond based *Artemia* culture systems, utilization of these associated bacteria may play a significant role. But in these open systems uncontrollable variations in many parameters, such as in the availability of different food sources (bacteria or algae) make it extremely difficult to evaluate the contribution of single diet among a wide variety of foods available at the same time. In laboratory co-feeding tests, *Artemia* has been proven to get more nutrients from bacteria in case of lack of microalgae supply (Toi et al., 2013), but only when isolating associated bacteria and offering them as food for *Artemia* under gnotobiotic culture conditions their nutritional role can be studied.

This study was therefore aiming at elucidating the effect of bacterial strains, isolated from *Artemia* cultures, on the performance of *Artemia* in terms of survival and body length and on nitrogen (N) assimilation in *Artemia*. For this purpose, both bacteria and algae were offered as mixed diets in different proportions to *Artemia* in gnotobiotic conditions. The ^{15}N stable isotope was used as a tool to measure N assimilation of either food source in the presence of the other, as this technique had been proven a valuable tool to assess protein assimilation in other studies (Preston et al., 1996; Burford et al., 2004; Avnimelech and Kochba, 2009). In addition, trypsin activity was measured to evaluate the digestive response in *Artemia* (Rojas-García et al., 2009) when bacteria and microalgae were offered together as food.

2.2. Materials and methods

2.2.1. Source of bacteria and microalgae

Nine bacteria strains coded from HT1 to HT9 were used in the experiment. These bacteria were isolated from the culture medium from a preliminary test run where *Artemia* had shown good performance in terms of survival and body length. In this preliminary *Artemia* culture baker's yeast *Saccharomyces cerevisiae* strain wild-type (WT) was offered to *Artemia*. The culture medium was spread in sterile marine agar (MA) 2216 plates (55.1 g/L; Difco™) and incubated in an incubator at 28 °C for 2-3 days. Colonies were distinguished by size, shape, color etc.; then a separated colony forming unit (CFU) was picked up by a sterile loop, streaked on other sterile MA plates (n = 2) and incubated in the same conditions. The CFU's uniform in size, color and shape were selected, then a single CFU was picked by sterile loop and inoculated in a sterile 50 mL Erlenmeyer with 20 mL of autoclaved marine broth (MB) 2216 (37.4 g/L; Difco™). Subsequently, the Erlenmeyer was closed with a sterile cotton cap. After inoculation, the culture was incubated in a shaker (28 °C; 150 rpm) for 24−48 h. When a visible color appeared, bacteria were harvested and stored in glycerol solution (20%) at − 80 °C for further use. Before feeding to *Artemia* nauplii, the purity of isolates was confirmed by denaturing gradient gel electrophoresis (DGGE).

Two axenic microalgae *Dunaliella tertiolecta* Butcher 1959 strains DT CCAP 19/6B (henceforth abbreviated DT 19/6B) and DT CCAP 19/27 (DT 19/27) were obtained from the Culture Collection of Algae and Protozoa Department (CCAP), Dunstaffnage Marine Laboratory, Scotland, UK. Marques et al. (2006b) confirmed that both *D. tertiolecta* strains are very similar in terms of DNA sequence: using the nuclear rRNA gene internal transcribed spacer region 2 showed that DT 19/6B (ASAP GeneBank accession number AY572957), and DT 19/27 (ASAP GeneBank accession number AY654300) are indeed both *D. tertiolecta*, though phenotypic differences have not been described (Marques et al., 2004a). The DT 19/6B strain had been proven as good-quality food for *Artemia,* resulting in 94% survival and 3.2 mm individual length after 6 days of culture (Marques et al., 2004a), and DT 19/27 as lower-quality food, (63.5% and 1.5 mm, respectively), for which reason they were included in our experiments.

2.2.2. Experimental design

2.2.2.1. Experiment 1: Effect of associated bacteria on *Artemia* performance

To evaluate the isolated bacteria as diet for *Artemia*, nine treatments were carried out on germ-free *Artemia* nauplii with the nine isolates. As positive and negative controls, *Artemia* were offered the bacterial strain LVS3 (*Aeromonas hydrophila*), or were starved, respectively. The reference bacterial strain LVS3 was used as positive control as it had proven positive effects on *Artemia* (Verschuere et al., 1999; Marques et al., 2005). The performance of *Artemia* in terms of survival, individual body length and total length was used as criterion to assess the positive effect of bacteria on *Artemia*. Twenty bacteria-free *Artemia* nauplii were transferred under laminar flow conditions to a sterile 40 mL screw-cap glass tube containing 30 mL of filtered (0.2 µm) autoclaved artificial seawater (FAASW) containing 30 g/L of Instant Ocean® synthetic sea salt. There were four replicates for each treatment. *Artemia* were daily fed with the corresponding un-labeled bacterial strains according to the treatments at a pre-tested concentration of 5×10^6 cells/mL (Marques et al., 2006a) over a 6 days culture period. After feeding, the tubes were carefully closed and placed on a rotator at 4 cycles per min in a temperature-controlled room (28.0 ± 1.0 °C).

Bacteria resulting in positive *Artemia* performance in this study were selected to run the next test aiming to elucidate the effect of associated bacteria and microalgae, offered together as food source, on nitrogen (N) assimilation in *Artemia.*

2.2.2.2. Experiment 2: Effect of presence of microalgae and bacteria on nitrogen assimilation in *Artemia*

Bacteria, for their positive effect on *Artemia* performance in term of growth and survival in the first experiment, have been chosen for current investigation. Two experiments were conducted to determine the contribution of bacteria versus microalgae on nitrogen assimilation in *Artemia* nauplii. In experiment 2a, aiming to measure the N assimilation from bacteria in the presence of microalgae, the ^{15}N labeled bacteria were fed together with an unlabeled microalgae strain, DT 19/6B or DT 19/27, resulting in four dietary combinations. For each combination bacteria-algae, different feeding tests were run corresponding with different proportions of

each food source. Analogously in the inverse experiment 2b, the ^{15}N labeled microalgae strains, DT 19/6B or DT 19/27, were fed to *Artemia* together with an unlabeled bacteria strain.

Hatched bacteria-free *Artemia* nauplii (second instar) were manipulated under the laminar flow hood for the experimental set-up. The nauplii were first harvested on a sterile sieve and washed thoroughly with FAASW to get rid of hatching waste products. *Artemia* nauplii were then diluted by FAASW in a sterile 500 mL beaker. Sub-samples of *Artemia* were collected (n = 4) to determine their density in 1 mL. Afterwards, the *Artemia* nauplii were distributed in 1 L sterile screw-cap bottles containing 1 L of FAASW at a stocking density of 20 nauplii/mL, and 9.1 mg (dry matter intake) of food was offered to each bottle, based on the feeding regime for *Artemia* as described by Coutteau et al. (1990). The proportion of each food in the mixed diets was prepared according to Table 2.1. In addition to the different proportions supplied (90/10, 50/50 and 10/90), cultures with the pure diets (100% microalgae or 100% bacteria) were used as controls. Three replicates were conducted for each treatment and for the controls. After feeding manipulation, the culture bottles were tightly closed by special screw caps supplied with an air inlet and outlet.

Table 2.1: Feeding regime for *Artemia* (adapted from Coutteau et al., 1990), expressing different portions (%) of each food type (bacteria versus microalgae) based on dry matter. Experiment 2a: *Artemia* fed on mixed diets consisting of ^{15}N labeled bacteria and unlabeled microalgae (DT 19/6B or DT 19/27). Experiment 2b: *Artemia* fed on mixed diets of ^{15}N labeled microalgae (^{15}N DT 19/6B or ^{15}N DT 19/27) and unlabeled bacteria.

Treatment code	^{15}N microalgae	bacteria	^{15}N bacteria	microalgae
Experiment 2a				
2A-FR1 (control 1)	0	0	100	0
2A-FR2	0	0	90	10
2A-FR3	0	0	50	50
2A-FR4	0	0	10	90
2A-FR5 (control 2)*	0	0	0	100
Experiment 2b				
2B-FR1 (control 1)	100	0	0	0
2B-FR2	90	10	0	0
2B-FR3	50	50	0	0
2B-FR4	10	90	0	0
2B-FR5 (control 2)*	0	100	0	0

*: the ^{15}N natural abundance of *Artemia* fed solely unlabeled microalgae (Experiment 2a) or unlabeled bacteria (Experiment 2b) was used as a background to calculate the excess of ^{15}N in *Artemia*.

The cultures were run in darkness to prevent uptake of nitrogen from the culture medium by the microalgae and lasted 24 h. Rifamycine (Sigma), as bacteriostatic agent, was added to each culture at a concentration of 0.005 g/L; preliminary tests using different Rifamycine concentrations had shown that this concentration was efficient in suppressing bacterial growth while having no effect on *Artemia* performance. The cultures were partially submerged in a temperature-controlled water bath at 28 °C with continuous 0.2 µm filtered aeration. After the 24 h culture period, *Artemia* nauplii were harvested on a sieve and washed gently as much as possible in FAASW to get rid of un-eaten feed and waste.

2.2.3. Diet preparation

2.2.3.1. Culturing, labeling and harvesting of microalgae

Microalgae were axenically grown in sterile 2 L bottles. Each bottle contained 2 L of FAASW enriched with 1 mL of standard Walne´s medium, supplied with 0.2 µm filtered aeration at 19 °C and continuous light (± 41 µE/m^2S) (Marques et al., 2004a). For labeling the microalgae, 0.01 g/L ^{15}N $NaNO_3$ (98 atom%, Sigma) was used to tag the algal proteins. Microalgae were harvested at the exponential growth phase by centrifuging. The algal cell pellet was washed twice in FAASW. According to Sorgeloos (1974) algae store enough photo-energy temporarily allowing cell division when transferred from light to darkness. Therefore, after harvesting the microalgae cell pellets were suspended in FAASW and the algal solution was stored at 4 °C for 24 h in darkness for cells to complete cell division before counting and offering to *Artemia*. The concentration of the algal suspension was determined by cell counting using a Burker haemocytometer chamber. After a 6-10 days growth period, the ^{15}N isotope signature in microalgae was 0.3696% and 0.3661% for non-^{15}N-enriched DT 19/27 and DT 19/6B, respectively. For ^{15}N-enriched microalgae, the ^{15}N isotope signature was 19.3770% and 23.7739% for DT 19/27 and DT 19/6B, respectively.

2.2.3.2. Culturing, labeling and harvesting of bacteria

For mass culturing, each strain of bacteria, stored at − 80 °C was streaked on MA (BD Difco™) plates (n = 2). Visible colonies appeared after two days of incubation at 28 °C. A single colony from each bacterial strain was picked by a sterile loop and transferred to a sterile Erlenmeyer containing autoclaved modified marine broth (M-MB). Briefly, M–MB was prepared using 0.1 g/L $NaNO_3$, 2.0 g/L yeast extract, 0.1 g/L Fe(III) citrate, 19.45 g/L NaCl, 5.9 g/L $MgCl_2$ (anhydrous), 3.24 g/L $NaSO_4$, 1.8 g/L $CaCl_2$, 0.55 g KCl, 0.16 g/L Na_2CO_3, 0.08 g/L KBr, 0.034 g/L $SrCl_2$, 0.022 g/L H_3BO_3, 0.004 g/L Na-silicate, 0.0024 g/L NaF, 0.0016 g/L NH_4NO_3, 0.008 g/L Na_2PO_4, with a final pH of 7.6. For labeling the bacteria, the M-MB ingredients were identical but 0.01 g/L $NaNO_3$ was replaced by ^{15}N $NaNO_3$ (Sigma). After inoculation, the inoculated flasks were carefully closed by sterile cotton caps and incubated in a shaker (28 °C; 150 rpm) for 24−48 h. The labeling technique resulted in an increase of ^{15}N signature in bacterial cells from 0.3677% to 6.5269% and from 0.3670% to 10.1780% for HT6 and HT3 bacteria, respectively.

When visible growth appeared, the bacterial suspension in the incubated flasks was transferred to sterile 50 mL screw-cap falcon tubes under laminar flow hood conditions. The cells were harvested at the stationary growth phase by centrifugation (± 4,400 × *g*; 15 min). Bacterial cell pellets were washed twice in FAASW. The bacterial cell pellets were then re-suspended in FAASW and the density of bacteria was determined by measuring its turbidity using a spectrophotometer set at 550 nm, assuming that an optical density of 1 corresponds to 1.2×10^9 cells/mL, according to the McFarland standard (BioMerieux, Marcy L'Etoile, France).

The dry weight (DW) of bacteria and microalgae was determined according to the methodology described by Soltanian et al. (2007). Briefly, 50 mL of culture suspension of each microalgae and bacteria strain was filtered through a pre-weighed 0.45 µm Whatman membrane using a Buchner funnel connected to a vacuum pump. The filter was then washed with ammonium formate solution (0.5 M) to remove the salt, placed into an aluminum pre-tared cup and dried in an oven at 104 ± 1 °C for 4 h. The samples were then removed from the oven, placed in a desiccator for cooling down, and weighed with an analytical balance (precision 0.1 mg).

2.2.4. Preparation of *Artemia* nauplii

For preparation of bacteria-free nauplii, cysts of *Artemia franciscana* Kellogg 1906 (EG® Type, INVE Aquaculture, Belgium) were decapsulated according to the protocol as described by Sorgeloos et al. (1977) and Marques et al. (2006a). Briefly, the dry cysts were first soaked in tap water for 1 h and then transferred to a laminar flow hood for decapsulation. The *Artemia* cyst shell was removed by reaction with sodium hypochlorite (NaOCl). Decapsulated cysts were harvested on a sterile sieve, and washed with FAASW as much as possible to remove all residual bleach. Decapsulated cysts were transferred into 1 L sterile bottles containing 1 L FAASW (Marques et al., 2006a) for hatching at 28 °C for 24-30 h under standardized hatching conditions (Sorgeloos et al., 1986).

2.2.5. Method used to verify axenity

Axenity of food*, Artemia* nauplii and *Artemia* culture medium was checked by the methodology as described by Marques et al. (2004a; 2004b). Briefly, food, *Artemia*, hatching and culture water were checked for contamination by plating on MA plates

(n = 2). The plates were checked for absence of bacteria after incubation at 28 °C for 5 days. The experiment was discarded whenever the *Artemia* nauplii, water or food were found to be contaminated.

2.2.6. Sample collection and data analysis

2.2.6.1. Survival, individual length and total biomass production

The data of survival, individual length (IL) and total length (TL) of *Artemia* were recorded only for experiment 1. At the end day of the culture period, free-swimming *Artemia* were counted and the survival of *Artemia* per treatment was calculated. Subsequently, the *Artemia* were fixed in Lugol´s solution to measure the IL, which was done using a dissecting microscope set with a drawing mirror, digital plan measure and the software *Artemia* 1.0 (courtesy of Marnix Van Damme). TL per tube was calculated according to the following equation as reported by Marques et al. (2006a):

Total length (mm/tube) = number of survivors x mean IL (mm)

2.2.6.2. The accumulation of nitrogen from food in *Artemia*

To prevent interference of undigested ^{15}N labeled food in the digestive tract of *Artemia* with the results of ^{15}N accumulation in *Artemia* tissue, the undigested food was biologically evacuated from the gut before ^{15}N analysis. This was done by placing the *Artemia* in a 1 L beaker containing 500 mL of FAASW and cellulose particles (20 µm; Sigma). The cellulose powder had been diluted in FAASW and sieved through a 50 µm net to prevent particle clumping. Cellulose was provided to the beakers with *Artemia* at a concentration three times higher than the feeding ration. Aeration was provided continuously to ensure homogeneous distribution of the cellulose in the water. *Artemia* was regularly checked under a binocular microscope for observation of the ingestion status. *Artemia* were harvested when the digestive tract was completely filled with cellulose.

After the evacuation step, *Artemia* were harvested on a sieve and rinsed with FAASW to remove all uneaten cellulose and wastes. After washing, *Artemia* was first soaked in a benzocaine solution (Sigma, 0.1%) for 10 s and then transferred to a benzalkonium chloride solution (Sigma, 0.1%) for 10 s to kill all the attached bacteria on the exoskeleton (Chládková et al., 2004). Afterwards, *Artemia* was washed as

much as possible with de-ionized water (DEMI-water). *Artemia* were then quickly stored in a freezer (− 20 °C) to prevent leaking of ^{15}N caused by the metabolism.

After thawing, *Artemia* were placed into a Petri dish with DEMI-water, and 200 to 600 *Artemia* nauplii from each replicate were sampled by a Pasteur pipette and rinsed on a metal mesh sieve (250 µm pore size). *Artemia* were then gently transferred using a small forceps onto a pre-weighed tin capsule cup of 8 x 5 mm. The wet samples were oven-dried at 70 °C for a day (De Troch et al., 2007), placed in the desiccator for cooling and weighed with an analytical balance (precision 0.1 mg). Subsequently, the ^{15}N assimilation in *Artemia* was measured by using an elemental analyzer (ANCA-SL, PDZ Europa, UK) coupled to an isotope-ratio mass spectrometer (IRMS) (20-20, SerCon, UK) at the Department of Applied Analytical and Physical Chemistry, Ghent University, Belgium.

The amount of nitrogen assimilation from food in *Artemia* (ng N/individual) was calculated with the equation described by Burford et al. (2004)**.**

N (ng/individual) = (e x n)/(f x no. *Artemia*)

where e: ^{15}N-ratio (atom % excess) in *Artemia*, n: total amount of nitrogen content in *Artemia* (ng), f: ^{15}N-ratio (atom % excess) in the food, no. *Artemia*: number of *Artemia*

2.2.6.3. Trypsin assay

To prepare crude enzyme extracts, *Artemia* samples (35 mg) from all treatments in experiment 2b were ground with a plastic pestle (25 s) in an eppendorf tube containing 1 mL of cold homogenizing mixture (1:1) of 0.85% NaCl-2.5 mM ethylenediaminetetraacetic acid (EDTA) and Trixton-X 1%-10 mM $CaCl_2$ (Rojas-García et al., 2009). After 24 h in darkness at 4 °C to allow enzyme extraction, the homogenates were centrifuged at 12,000 x *g* for 30 min at 4 °C. The supernatant was used as crude enzyme extract. Trypsin-L activity was measured using N-alpha-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Tseng et al., 1982) as the substrate in 50 mM Tris-HCl and 10 mM $CaCl_2$ buffer, pH 7.1. The measurement and calculation of trypsin activity was done by the methodology as described by Rojas-García et al. (2009). Briefly, the p-nitroanilide (pNA) formation by enzymatic BAPNA breakdown was recorded spectrophotometrically after 24 h. DO_{410} increment (Δ) was calculated as the difference of DO_{410} at 24 h (DO_f) and at time 0 h (DO_i). Background

DO_{410} (increment) due to non-enzymatic pNA production was assayed in tubes without crude enzyme extract using buffer and BAPNA.

$\Delta_{\text{sample or background}} = DO_f - DO_i$

The DO_{410} increments due to positive trypsin-L were calculated by subtraction as follows:

Trypsin-L (ΔDO_{410}) = $\Delta_{\text{sample}} - \Delta_{\text{background}}$.

2.2.7. Statistical analysis

The data of nitrogen assimilation and trypsin-L were checked for homogeneity of variance and normal distribution by Levene's *F* test and P-P plot by using Statistica 7.0 for Windows. The data failed to meet these assumptions and were logarithmically transformed to satisfy normal distribution and to homogenize variance. 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.

2.3. Results

2.3.1. The effect of isolated bacterial strains on *Artemia* performance

Survival, IL and TL of *Artemia* fed the reference strain LVS3 and the experimental bacteria strains are listed in Table 2.2. Among 9 strains of tested bacteria, only HT3, HT6, HT7 and HT8 yielded some survival. The highest survival was reported in HT6, which was even higher than the survival of the reference bacteria LVS3. The HT3 strain also showed a survival comparable to that of the reference bacteria, whereas all others were inferior in survival than the reference. The individual length also followed a similar pattern, where the highest value was recorded in HT3, while HT6 was comparable to that of HT3, but both of them were not significantly different ($p > 0.05$) from the reference LVS3. Similarly, TBP was higher in both HT3 and HT6 as compared to the reference bacteria, though not significantly. The highest TL was recorded in HT6.

Table 2.2: Average survival (%), individual length IL (mm) and total length TL (mm/tube) of *Artemia* fed on bacteria isolated from *Artemia* cultures. The values are mean ± standard deviation (n = 4). Different superscripts in the same column denote significant differences ($p < 0.05$). IL values showed no significant differences.

Treatment	Survival (%)	IL (mm)	TL (mm)/tube
1. LVS3 (control)	35 ± 11^{c}	0.9 ± 0.2	6.4 ± 2.2^{b}
2. No bacteria	0.0^{a}	-	-
3. HT1	0.0^{a}	-	-
4. HT2	0.0^{a}	-	-
5. HT3	35 ± 4^{c}	1.1 ± 0.2	7.5 ± 1.3^{b}
6. HT4	0.0^{a}	-	-
7. HT5	0.0^{a}	-	-
8. HT6	48 ± 14^{c}	1.0 ± 0.2	9.7 ± 3.4^{b}
9. HT7	6 ± 5^{b}	0.9 ± 0.1	1.1 ± 0.9^{a}
10. HT8	13 ± 10^{bc}	1.0 ± 0.2	2.5 ± 2.1^{a}
11. HT9	0.0^{a}	-	-

2.3.2. Effect of the presence of microalgae on N assimilation from bacteria in *Artemia*

Assimilation of N from HT6 was evidently higher than from HT3 in 100% bacteria-fed *Artemia*. The N assimilation from either HT6 or HT3 was not reduced proportionately with the reduction of HT3 or HT6 in the mixed diets with microalgae (Fig. 2.1). In the case of co-feeding with DT 19/27, N assimilation for either HT3 or HT6 steadily reduced with the reduction of the amount of bacteria from 100% to 10%. For HT3 and HT6 this reduction was significantly different ($p < 0.05$) from the respective controls (100% bacteria) from 50% and 90% replacement with DT 19/27, respectively, onwards. In contrast, co-feeding with DT 19/6B first increased assimilation of N from either HT3 or HT6 compared to the respective controls (100% bacteria), and this increase was significant ($p < 0.05$) when HT6 was replaced for 10% by DT 19/6B. For both bacterial strains 90% replacement by DT 19/6B, however, resulted in a significant reduction ($p < 0.05$) of N assimilation compared to the control with 100% bacteria. Moreover, the combination with DT 19/6B always

resulted in higher N assimilation from any type of bacterial strain than the one with DT 19/27.

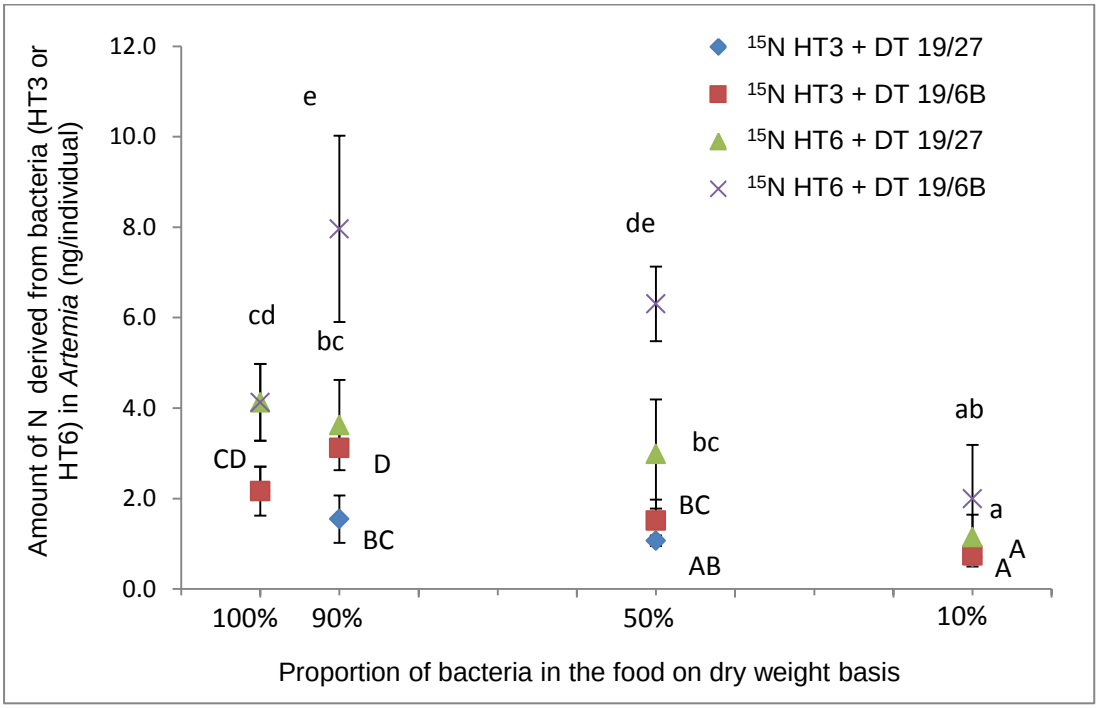

Figure 2.1: Effect of the presence of microalgae on nitrogen assimilation from bacteria (ng/individual) in *Artemia*. The values are mean ± standard deviation (n = 3). Different superscripts of HT3 treatments or HT6 treatments indicate significant difference ($p < 0.05$) between the treatments. For the proportion of bacteria in the mixed diets, see Table 2.1.

2.3.3. Effect of presence of bacteria on N assimilation from microalgae in *Artemia*

A higher N assimilation was always obtained from DT 19/6B than from DT 19/27 in the corresponding treatments (Fig. 2.2). Replacement of 90% of either alga by HT6 led to a significant reduction of N assimilation from the microalgae ($p < 0.05$) as compared to the control with 100% microalgae fed *Artemia.* Also replacement of 90% of microalgae by HT3 led to a significant reduction of N assimilation as compared to the control. The combination of microalgae with HT6 always resulted in higher N assimilation from the algae than with HT3.

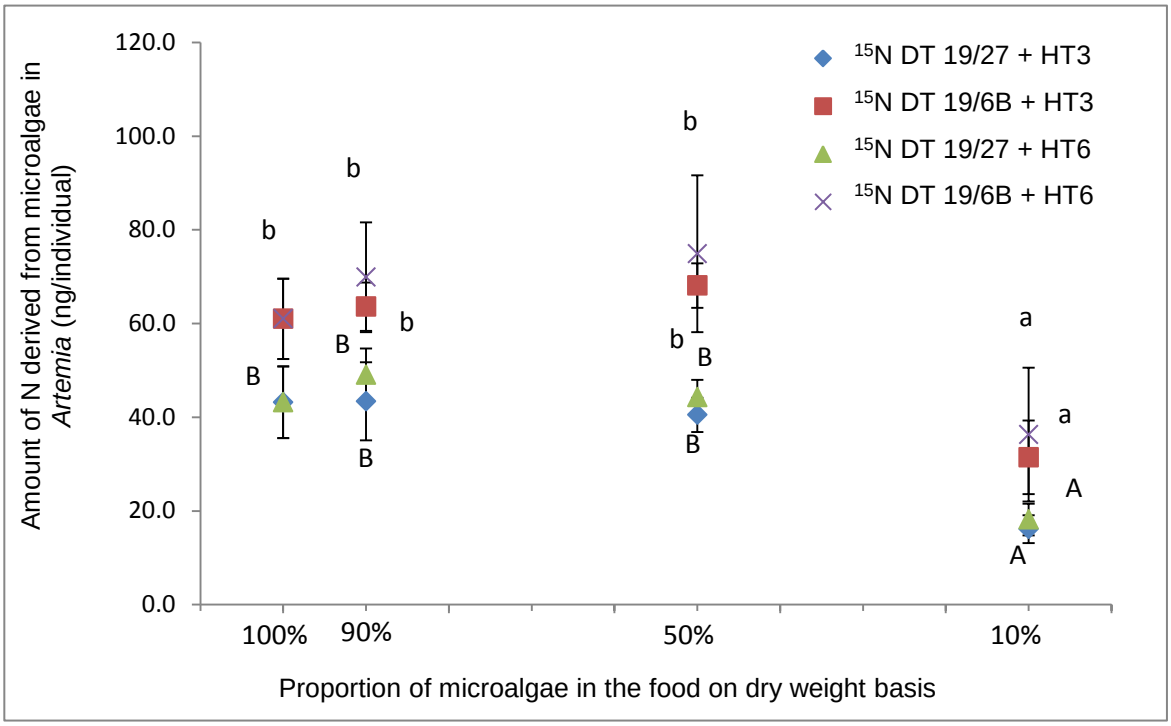

Figure 2.2: Effect of the presence of bacteria on nitrogen assimilation from microalgae (ng/individual) in *Artemia*. The values are mean ± standard deviation (n = 3). Different superscripts of DT 19/27 treatments or DT 19/6B treatments indicate significant difference ($p < 0.05$) between the treatments. For the proportion of microalgae in the mixed diets, see Table 2.1.

2.3.4. Trypsin activity of *Artemia* fed mixed diets of microalgae and bacteria

Trypsin-L activity in 100% microalgae fed *Artemia* was similar for both strains of microalgae DT 19/27 (Fig. 2.3 A) and DT 19/6B (Fig. 2.3 B) and was similar to the values obtained for 100% HT6 fed *Artemia* or for *Artemia* fed with any combination of algae and HT3 or HT6. Only the values for 100% HT3 fed *Artemia* were significantly lower ($p < 0.05$) than in the other feeding regimes.

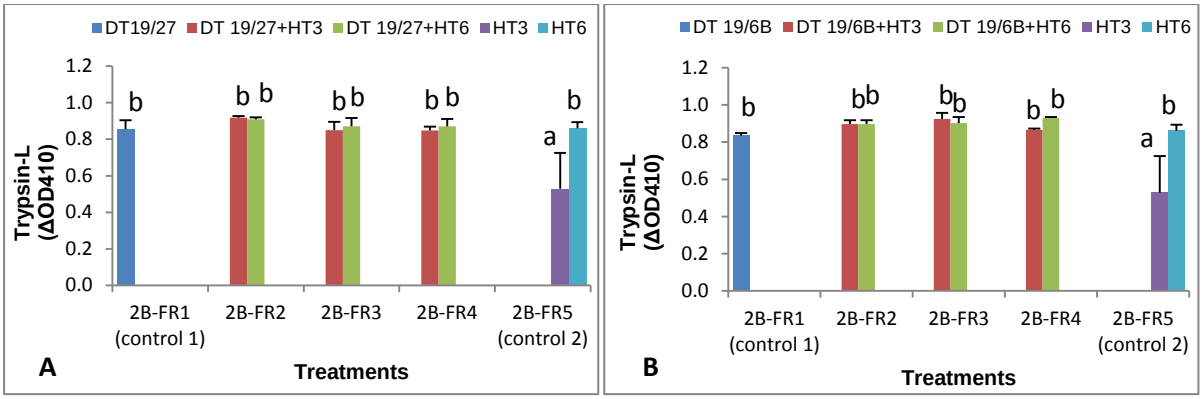

Figure 2.3: Effect of DT 19/27 (A) and DT 19/6B (B) diets (pure and mixed with bacteria) on trypsin-L activity in *Artemia* nauplii. Values are mean ± standard deviation (n = 3). Different superscripts within each figure indicate significant difference ($p < 0.05$) between the treatments. For abbreviation of the treatments, see Table 2.1. Also trypsin-L values of control 2 (100% HT3 or HT6) are shown on each graph.

2.4. Discussion

In our current study, to investigate whether associated bacteria have any effect towards *Artemia*, gnotobiotic culture conditions were used. In the first set of culture tests, the isolates, originated from *Artemia* culture, were offered to *Artemia* nauplii as food source. A positive effect of some isolates on *Artemia* survival was observed, e.g. *Artemia* nauplii could survive and grow on the HT3, HT6, HT7 and HT8 bacterial strains. The survival of HT3 and HT6 fed *Artemia* was higher or identical to that obtained in *Artemia* fed the reference bacteria LVS3 (*Aeromonas hydrophila*), while the survival of HT7 and HT8 fed *Artemia*, however, was significantly lower than in the LVS3 fed *Artemia*. As a result, the total body length of HT6 and HT3 fed *Artemia* was higher (though not significant) than that obtained in reference LVS3 fed *Artemia*, although all bacteria were applied at the same concentrations and in the same culture conditions. These results suggest that the HT6 may provide more nutrients to *Artemia* nauplii as compared to HT3 and LVS3. Later HT3 and HT6 were sequenced and blasted with http://blast.ncbi.nlm.nih.gov and aligned in http://multalin.toulouse.inra.fr/. The HT3 and HT6 strains showed 99% similarity with *Tamlana* sp. ZJU HZ22 and *Bacillus subtilis* 168, respectively. *Artemia* thriving solely on bacteria have been reported from previous studies; for example *Artemia* was able to live on *Pseudomonas* sp. (Gorospe et al., 1996), B-9 (*Acinetobacter*

strain) (Yasuda and Taga, 1980) and LVS2 (*Bacillus* sp.), LVS3 and LVS8 (*Vibrio* sp.) (Verschuere et al., 2000; Marques et al., 2006a; 2006c). The usage of bacterial fatty acids (*Flexibacter* strain Inp3) in *Artemia* has been demonstrated by increased levels in *Artemia* of 16:1n-7 and 18:1n-7, the main major fatty acids in bacterial cells (Intriago and Jones, 1993).

However, the survival of *Artemia* was zero in the culture with the addition of the other bacteria strains and in the culture without the addition of bacteria. The total mortality of starved *Artemia* after 6 days in our study is in agreement with the observation by Treece (2000) and Marques et al. (2005) that starved *Artemia* metanauplii have consumed all yolk reserves and don't survive in the absence of external food beyond the 4^{th} after hatching (DAH). Apart from HT3 and HT6 no attempt was made to indentify these bacteria. May be they sustain *Artemia* growth for a period shorter than 6 days.

In a subsequent step, experiments were conducted aiming to elucidate the effect of associated bacteria and microalgae, offered together as food source, on N assimilation in *Artemia*. The bacteria strains HT3 and HT6 were selected based on their positive effect on growth and survival as described above. Two strains of the microalga *Dunaliella tertiolecta*, DT 19/6B and DT 19/27, with different quality as food for *Artemia*, were offered either as mono-diet or in mixed diets of bacteria and microalgae. As the experiment was conducted over just a short period (24 h) and as there were no obvious differences in *Artemia* length and survival among the treatments (results not shown) after 24 h, we assumed that there were no differences in food consumption by *Artemia*, which might have affected N assimilation. When microalgae were offered as mono-diet to *Artemia*, this study is the first to show that higher N assimilation was obtained from DT 19/6B as compared to DT 19/27. Marques et al. (2006b) reported that there are no records of genotypic or phenotypic difference between the DT 19/6B and DT 19/27 strains of *D. tertiolecta*, though *Artemia* fed on the former is always performing better than on the latter. This may related to the higher nutrient content of DT 19/6B (Marques et al., 2004a).

Our results of assimilation of ^{15}N, which was used to label bacteria (food) and which was found to accumulate in *Artemia* tissue, also confirm that *Artemia* can ingest and assimilate associated bacteria. The N assimilation from HT6 was always higher than

from HT3, although equal amounts (on dry weight basis) were offered to *Artemia*, probably due to the higher digestibility and/or nutrient levels in the former strain. Moore and Jaeckle (2010) stated that bacteria are not high-energy food for *Artemia* as compared to microalgae. The clearance rate (volume of cleared particles per unit of time) of *Artemia* larvae fed bacterial particles size (0.5 µm) is 69 times less than when fed algal particles size (12 µm) (Moore and Jaeckle, 2010). In our study N assimilation from the microalga *D. tertiolecta* in *Artemia* is more than 10 times higher than from bacteria, although equal amounts (on dry weight basis) of microalgae and bacteria were offered to *Artemia*. These results suggest that microalgae are an overall better nutritional source for *Artemia* than bacteria.

When bacteria were offered to *Artemia* together with microalgae, N assimilation from bacteria was firstly related to the degree of replacement of bacteria by microalgae, and secondly to the strain of microalgae. In mixed diets with any type of bacterial strains, the presence of DT 19/6B always induced better N assimilation from the bacteria than DT 19/27. At any proportion of bacteria in the food the N assimilation from either bacteria strain was higher with DT 19/6B than with DT 19/27 replacement. At 10% HT6 replacement by DT 19/6B, the N assimilation from either bacteria strain nearly doubled. A first possible explanation for this difference between both microalgae could be that the presence of DT 19/6B as replacement provides more digestive enzymes than replacement by DT 19/27. Berges and Falkowski (1996) reported that proteases are present in *D. tertiolecta* cells. The enzymes may be released from broken cells of DT 19/6B, and together with the matching enzymes of *Artemia* they may degrade bacteria making them easier to absorb by *Artemia*. Alternatively, the presence of microalgae may also trigger the digestion process by stimulating enzymatic activity in the *Artemia* gut. Also in sea bass *Dicentrarchus labrax* larvae the addition of microalgae in the rearing water has been demonstrated to induce the release of digestive enzymes (Cahu et al., 1998).

Our results further show that the presence of any type of bacteria slightly increased or was identical to the N assimilation from microalgae compared to solely microalgae fed *Artemia*, although the concentration of microalgae was reduced with 10% and 50% in the mixed diets.

The addition of live bacteria in the *Artemia* cultures may provide the host with exogenous extracellular enzymes such as inter- or extra-cellular β-glucosidase

(cellulolytic activity) as previously reported (Verschuere et al., 1999; Maki et al., 2009). Together with the host's enzymes, these enzymes may allow for better degradation of algal cells (Intriago and Jones, 1993; Sander and Murthy, 1999; Marques et al., 2005). Analogously it has been demonstrated that the administration of bacteria to juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) positively increased its enzymatic activity (Sáenz de Rodrigáñez et al., 2009).

Nevertheless, no obvious relationship was found between trypsin-L activities and N assimilation in *Artemia* in this study. Significantly higher trypsin-L activity was obtained in all treatments where *Artemia* was fed on mixed diets of bacteria and microalgae, as compared to solely HT3 fed *Artemia*, while there was no difference with solely HT6 fed *Artemia*, which confirms the lower nutritional value of HT3. But no significant differences in trypsin-L activity were found among the mixed diets and the 100% microalgae control for any type of microalgae used.

This study shows that, provided suitable strains are used, offering microalgae and bacteria together as food for *Artemia* may result in a beneficial effect in how this filter-feeding organism takes advantage of the respective food sources. This is of immediate relevance for *Artemia* laboratory cultures, where generally algae diets are used in xenic conditions, but also for *Artemia* pond production procedures, which emphasize the induction of phytoplankton blooms but overlook the role of the aquatic bacterial communities. Finally, our tests with *Artemia* can be considered as a model for future research on the feeding biology of other filter-feeding aquaculture organisms.

Acknowledgements

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Chapter 3

Application of a ^{15}N tracer method to study the complementarity of bacteria versus yeast in nitrogen assimilation by *Artemia* nauplii

Chapter 3: Application of a ^{15}N tracer method to study the complementarity of bacteria versus yeast in nitrogen assimilation by *Artemia* nauplii

Abstract

Bacteria naturally occurring in *Artemia* culture systems not only act as direct food for the culture organism, but are also a possible source of exogenous enzymes. However, studies in this regard have not gone beyond basic *Artemia* growth studies and morphological analysis of the digestive tract and speculations based on them. The present study therefore aimed at elucidating the possible effects of two bacterial isolates, HT3 and HT6, resulting in lower and higher *Artemia* survival and growth, respectively, and two baker´s yeast strains i.e. wild-type (WT) and its mutant mnn9 on nitrogen (N) assimilation of *Artemia*. Using the ^{15}N stable isotope technique, N assimilation of *Artemia* was determined in feeding regimes where the two yeast strains WT and mnn9 were gradually replaced by HT3 or HT6, or where the bacterial strains were gradually replaced by each yeast strain. A trypsin assay was performed on *Artemia* fed the various replacement feeding regimes, in order to detect the effect of the presence of bacteria or of the yeasts on the protein digestive response in *Artemia*.

HT6 fed *Artemia* showed a higher N assimilation than when using HT3, suggesting a better food value of HT6 for *Artemia*. The assimilation of nitrogen from bacteria was related to the presence of yeast strains. In the HT3 fed *Artemia* group, the presence of WT yeast showed higher N assimilation from HT3 than the presence of mnn9, suggesting an enhanced utilization of poor quality HT3 in the presence of WT yeast. When using the higher quality HT6 on the other hand, mnn9 acted in a very similar manner. This is the first observation where mnn9 improves the N assimilation from any bacteria. When N assimilation from yeast strains was tested in the presence of bacteria, both bacterial strains HT6 and HT3 showed a clear enhancement in utilization of N from mnn9, probably by providing exogenous enzymes, highlighting the additional importance of these strains of bacteria for *Artemia* in utilizing yeast. A significant reduction ($p < 0.05$) of trypsin activity was observed in *Artemia* fed HT3 alone, as compared to all other feeding treatments, confirming the lower suitability of

the HT3 isolate for sole feeding of *Artemia*. We conclude that HT6, being a better N source for *Artemia*, also favors the utilization of N derived from yeast in the food.

3.1. Introduction

Hatched *Artemia* nauplii have been one of the most reliable live food organisms for fish and shellfish larviculture ever since their use was reported for the first time many decades ago (Seale, 1933; Sorgeloos et al., 2001). The ability of *Artemia* to thrive well both in controlled indoor conditions and in exposed outdoor conditions is considered invaluable for a variety of its applications. *Artemia* is extensively cultured in typical outdoor conditions such as saltworks in the Mekong delta, Vietnam. Animal waste material and agricultural byproducts have reportedly been used for this type of pond production, either for the purpose of manuring the ponds to stimulate primary production and/or for direct feeding (Brands, 1996; Baert et al., 1997; Anh, 2009).

Both in outdoor and indoor production systems, bacteria in the water play a role in *Artemia* nutrition. Bacteria in culture systems prove to be a source of food for the filter feeding *Artemia* (Yasuda and Taga, 1980; Douillet, 1987; Intriago and Jones, 1993; Gorospe et al., 1996), e.g. as a dietary source of fatty acids and amino acids (Austin, 1988; Gorospe et al., 1996). Moreover, bacterial enzymes act as extrinsic enzymes in the *Artemia* gut, facilitating the digestive capability of the host (Intriago and Jones, 1993; Marques et al., 2004a). Bacteria also remove toxic metabolites (Verschuere et al., 2000) by the bacterial nitrification process in which ammonium is converted to nitrite and then to the less toxic nitrate. The current knowledge about the function or the use of bacteria as a food resource for *Artemia* is limited to studies relating bacterial ingestion with *Artemia* growth. The function of bacteria in *Artemia* nutrition at physiological levels such as food assimilation, however, largely remains unknown.

The present study was therefore conducted to elucidate the contribution of two known bacterial isolates from a laboratory *Artemia* culture (see chapter 2) to nitrogen assimilation in *Artemia* when they were used in a controlled laboratory culture system alongside two strains of baker´s yeast *Saccharomyses cerevisiae* (wild type and mnn9), assumed to have different digestibility by *Artemia*, resulting in different *Artemia* growth. The latter strain has reduced mannose and increased glucan content in the cell wall as compared to the wild type strain. Coutteau et al. (1990)

detected β-glucanase activity in the *Artemia* gut, but no mannase activity. In addition, trypsin activity was measured to evaluate the digestive response in *Artemia* (Rojas-García et al., 2009) when bacteria and yeast were offered together as food. The ^{15}N tracer method was used as a tool to detect the exact nitrogen source of the protein assimilated.

3.2. Materials and methods

3.2.1. Experimental design

Two experiments were performed under gnotobiotic culture conditions; the first to determine the effect of the presence of yeasts on nitrogen derived from bacteria in *Artemia* nauplii, and the second to determine the effect of the presence of bacteria on nitrogen derived from yeasts.

Hatched bacteria-free *Artemia* nauplii (second instar) were manipulated under the laminar flow hood for the experimental set-up. The nauplii were first harvested on a sterile sieve and washed thoroughly with FAASW to get rid of hatching waste products. *Artemia* nauplii were then diluted by FAASW in a sterile 500 mL beaker. Sub-samples of *Artemia* were collected (n = 4) to determine their density in 1 mL. Afterwards, the *Artemia* nauplii were distributed in 1 L sterile screw-cap bottles containing 1 L of FAASW at a stocking density of 20 nauplii/mL, and 9.1 mg (dry matter intake) of food was offered to each bottle, based on the feeding regime for *Artemia* as described by Coutteau et al. (1990). The proportion of each food in the mixed diets was prepared according to Table 3.1.

In experiment 1, *Artemia* were fed mixed diets containing the ^{15}N labeled bacterial strain HT3 together with unlabeled WT or mnn9 yeast. In another set of treatments *Artemia* were fed mixed diets containing the ^{15}N labeled bacterial strain HT6 and unlabeled yeast WT or mnn9. In experiment 2, labeled WT and mnn9 yeast were fed to *Artemia* together with the unlabeled bacterial strains HT3 or HT6. In both experiments, yeasts and bacteria were offered to *Artemia* in different proportions (90/10, 50/50 and 10/90), and two treatments with the pure diet (100% yeasts or 100% bacteria) were used as controls. After feeding manipulation, the culture bottles were tightly closed by special screw caps supplied with an air inlet and outlet. Three replications for each treatment and for the control were run under gnotobiotic conditions for 24 h according to the methodology as described in chapter 2.

Table 3.1: Feeding regime for *Artemia* nauplii during 24 h (adapted from Coutteau et al. 1990) expressing different portions (%) of each food type (bacteria versus yeast). based on dry matter. Experiment 1: *Artemia* fed mixed diets consisting of unlabeled yeast (WT or mnn9) and ^{15}N labeled bacteria (^{15}N HT3 or ^{15}N HT6). Experiment 2: *Artemia* fed mixed diets consisting of ^{15}N labeled yeast (^{15}N WT or ^{15}N mnn9) and unlabeled bacteria (HT3 or HT6)

Treatment code	^{15}N yeasts	Bacteria	^{15}N bacteria	Yeasts
Experiment 1				
E1-FR1 (control 1)	0	0	100	0
E1-FR2	0	0	90	10
E1-FR3	0	0	50	50
E1-FR4	0	0	10	90
E1-FR5 (control 2)*	0	0	0	100
Experiment 2				
E2-FR1 (control 1)	100	0	0	0
E2-FR2	90	10	0	0
E2-FR3	50	50	0	0
E2-FR4	10	90	0	0
E2-FR5 (control 2)*	0	100	0	0

*: ^{15}N natural abundance of *Artemia* fed solely unlabeled yeasts (Experiment 1) or unlabeled bacteria (Experiment 2) was used as a background to calculate the excess of ^{15}N and N assimilation in *Artemia*.

3.2.2. Preparation of *Artemia* nauplii [1]

For preparation of bacteria-free nauplii, cysts of *Artemia franciscana* Kellogg 1906 (EG® Type, INVE Aquaculture, Belgium) were decapsulated according to the protocol as described by Sorgeloos et al. (1977) and Marques et al. (2006a). Briefly, the dry cysts were first soaked in tap water for 1 h and then transferred to a laminar flow hood for decapsulation. The *Artemia* cyst shell was removed by reaction with sodium hypochlorite (NaOCl). Decapsulated cysts were harvested on a sterile sieve, and washed with FAASW as much as possible to remove all residual bleach.

[1] See 2.2.4

Decapsulated cysts were transferred into 1 L sterile bottles containing 1 L FAASW (Marques et al., 2006a) for hatching at 28 °C for 24 − 30 h under standardized hatching conditions (Sorgeloos et al., 1986).

3.2.3. Diet preparation

3.2.3.1. Culturing, labeling and harvesting of yeast

Two baker´s yeast strains i.e. wild-type (WT) and its mutant mnn9, originated from the European *Saccharomyces cerevisiae* archive for functional analysis EUROSCARF, University of Frankfurt, Germany, were used in the study. WT was used for its low digestibility and the strain mnn9 for its improved digestibility by *Artemia* (Marques et al., 2004b).

Before mass culturing, yeast was streaked on a Yeast Extract Peptone Dextrose (YEPD) agar plate (n = 2) containing 10 g/L yeast extract (Sigma), 10 g/L peptone (Sigma), 20 g/L dextrose (Sigma), and 20 g/L agar. The medium was prepared in FAASW. Visible colonies appeared after three days incubation in the dark at 28 °C. A single colony forming unit (CFU) of each yeast strain was picked up from the agar plate by a sterile loop and transferred into 500 mL sterile Erlenmeyer flasks for inoculation. Each inoculated Erlenmeyer contained 300 mL of 0.2 µm filter-sterilized yeast nitrogen base medium (YNB, without ammonium sulfate and amino acids), and was supplemented with 5.0 g/L ammonium sulfate, 5.0 g/L D-glucose, 0.02 g/L L-histidine, 0.04 g/L L-methionine, and 0.04 g/L LD-tryptophan in FAASW. For ^{15}N yeast enrichment, each strain of yeast was grown on sterile YNB without ammonium sulfate and amino acid medium, supplemented with 4.99 g/L ammonium sulfate (Sigma) and 0.01 g/L ^{15}N NH_4Cl (Sigma) and the concentration of each amino acid was similar to that of the unlabeled yeast cultures.

After inoculation, the inoculated flasks were carefully closed by sterile cotton caps and incubated in a shaker (28 °C; 120 rpm). Yeast cultures were harvested in the exponential growth phase, detected by optical density (Marques et al., 2004a). Yeast cultures from the Erlenmeyer were first transferred to 50 mL sterile screw-cap plastic falcon tubes, and the yeast cell pellet was obtained by centrifuging (± 2,000 x *g*; 5 min). The pellet was washed twice in FAASW (Marques et al., 2006a; Soltanian et al., 2007) and then resuspended in FAASW. The yeast solution was stored at 4 °C for subsequent use. Its concentration was determined based on cell counts using a

Burker haemocytometer chamber. All the manipulations related to yeast harvesting were performed under a laminar flow hood to maintain sterility. After a 24–36 h growth period, the ^{15}N signature increased from 0.3709% to 1.8832% and from 0.3707% to 1.3796% for WT and mnn9 yeast, respectively.

3.2.3.2. Culturing, labeling and harvesting of bacteria[2]

For mass culturing, each strain of bacteria, stored at − 80 °C was streaked on MA (BD Difco™) plates (n = 2). Visible colonies appeared after two days of incubation at 28 °C. A single colony from each bacterial strain was picked by a sterile loop and transferred to a sterile Erlenmeyer containing autoclaved modified marine broth (M-MB). Briefly, M–MB was prepared using 0.1 g/L $NaNO_3$, 2.0 g/L yeast extract, 0.1 g/L Fe(III) citrate, 19.45 g/L NaCl, 5.9 g/L $MgCl_2$ (anhydrous), 3.24 g/L $NaSO_4$, 1.8 g/L $CaCl_2$, 0.55 g KCl, 0.16 g/L Na_2CO_3, 0.08 g/L KBr, 0.034 g/L $SrCl_2$, 0.022 g/L H_3BO_3, 0.004 g/L Na-silicate, 0.0024 g/L NaF, 0.0016 g/L NH_4NO_3, 0.008 g/L Na_2PO_4, with a final pH of 7.6. For labeling the bacteria, the M-MB ingredients were identical but 0.01 g/L $NaNO_3$ was replaced by ^{15}N $NaNO_3$ (Sigma). After inoculation, the inoculated flasks were carefully closed by sterile cotton caps and incubated in a shaker (28 °C; 150 rpm) for 24−48 h. The labeling technique resulted in an increase of ^{15}N signature in bacterial cells from 0.3677% to 8.7586% and from 0.3670% to 9.1965% for HT6 and HT3 bacteria, respectively.

When visible growth appeared, the bacterial suspension in the incubated flasks was transferred to sterile 50 mL screw-cap falcon tubes under laminar flow hood conditions. The cells were harvested at the stationary growth phase by centrifugation (± 4,400 × *g*; 15 min). Bacterial cell pellets were washed twice in FAASW. The bacterial cell pellets were then re-suspended in FAASW and the density of bacteria was determined by measuring its turbidity using a spectrophotometer set at 550 nm, assuming that an optical density of 1 corresponds to 1.2×10^9 cells/mL, according to the McFarland standard (BioMerieux, Marcy L'Etoile, France).

3.2.4. Dry weight determination of yeast and bacteria

Dry weight (DW) of yeast and bacteria was determined according to the methodology described by Soltanian et al. (2007). Briefly, 50 mL of culture suspension of each yeast and bacteria strain was filtered through a pre-weighed

[2] See 2.2.3.2

0.45 µm Whatman membrane using a Buchner funnel connected to a vacuum pump. The filter was then washed with ammonium formate solution (0.5 M) to remove the salt, placed into an aluminum pre-tared cup and dried in an oven at 104 ± 1 °C for 4 h. The samples were then removed from the oven, placed in a desiccator for cooling down, and weighed with an analytical balance (0.0001 g accuracy)

3.2.5. Sample collection and data analysis

3.2.5.1. Nitrogen accumulation in *Artemia*[3]

To prevent interference of undigested ^{15}N labeled food in the digestive tract of *Artemia* with the results of ^{15}N accumulation in *Artemia* tissue, the undigested food was biologically evacuated from the gut before ^{15}N analysis. This was done by placing the *Artemia* in a 1 L beaker containing 500 mL of FAASW and cellulose particles (20 µm; Sigma). The cellulose powder had been diluted in FAASW and sieved through a 50 µm net to prevent particle clumping. Cellulose was provided to the beakers with *Artemia* at a concentration three times higher than the feeding ration. Aeration was provided continuously to ensure homogeneous distribution of the cellulose in the water. *Artemia* was regularly checked under a binocular microscope for observation of the ingestion status. *Artemia* were harvested when the digestive tract was completely filled with cellulose.

After the evacuation step, *Artemia* were harvested on a sieve and rinsed with FAASW to remove all uneaten cellulose and wastes. After washing, *Artemia* was first soaked in a benzocaine solution (Sigma, 0.1%) for 10 s and then transferred to a benzalkonium chloride solution (Sigma, 0.1%) for 10 s to kill all the attached bacteria on the exoskeleton (Chládková et al., 2004). Afterwards, *Artemia* was washed as much as possible with de-ionized water (DEMI-water). *Artemia* were then quickly stored in a freezer (− 20 °C) to prevent leaking of ^{15}N caused by the metabolism.

After thawing, *Artemia* were placed into a Petri dish with DEMI-water, and 200 to 600 *Artemia* nauplii from each replicate were sampled by a Pasteur pipette and rinsed on a metal mesh sieve (250 µm pore size). *Artemia* were then gently transferred using a small forceps onto a pre-weighed tin capsule cup of 8 x 5 mm. The wet samples were oven-dried at 70 °C for a day (De Troch et al., 2007), placed in the desiccator for cooling and weighed with an analytical balance (precision 0.1 mg). Subsequently,

[3] See 2.2.6.2

the ^{15}N assimilation in *Artemia* was measured by using an elemental analyzer (ANCA-SL, PDZ Europa, UK) coupled to an isotope-ratio mass spectrometer (IRMS) (20-20, SerCon, UK) at the Department of Applied Analytical and Physical Chemistry, Ghent University, Belgium.

The amount of nitrogen assimilation from food in *Artemia* (ng N/individual) was calculated with the equation described by Burford et al. (2004)**.**

N (ng/individual) = (e x n)/(f x no. *Artemia*)

where e: ^{15}N-ratio (atom % excess) in *Artemia*, n: total amount of nitrogen content in *Artemia* (ng), f: ^{15}N-ratio (atom % excess) in the food, no. *Artemia*: number of *Artemia*.

3.2.5.2. Trypsin assay [4]

To prepare crude enzyme extracts, *Artemia* samples (35 mg) from all treatments in experiment 2 were ground with a plastic pestle (25 s) in an eppendorf tube containing 1 mL of cold homogenizing mixture (1:1) of 0.85% NaCl-2.5 mM ethylenediaminetetraacetic acid (EDTA) and Trixton-X 1%-10 mM $CaCl_2$ (Rojas-García et al., 2009). After 24 h in darkness at 4 °C to allow enzyme extraction, the homogenates were centrifuged at 12,000 x *g* for 30 min at 4 °C. The supernatant was used as crude enzyme extract. Trypsin-L activity was measured using N-alpha-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Tseng et al., 1982) as the substrate in 50 mM Tris-HCl and 10 mM $CaCl_2$ buffer, pH 7.1. The measurement and calculation of trypsin activity was done by the methodology as described by Rojas-García et al. (2009). Briefly, the p-nitroanilide (pNA) formation by enzymatic BAPNA breakdown was recorded spectrophotometrically after 24 h. DO_{410} increment (Δ) was calculated as the difference of DO_{410} at 24 h (DO_f) and at time 0 h (DO_i). Background DO_{410} (increment) due to non-enzymatic pNA production was assayed in tubes without crude enzyme extract using buffer and BAPNA.

$\Delta_{sample\ or\ background} = DO_f - DO_i$

The DO_{410} increments due to positive trypsin-L were calculated by subtraction as follows:

Trypsin-L (ΔDO_{410}) = $\Delta_{sample} - \Delta_{background.}$

[4] See 2.2.6.3

3.2.6. Method used to verify axenity [5]

Axenity of food*, Artemia* nauplii and *Artemia* culture medium was checked by the methodology as described by Marques et al. (2004a; 2004b). Briefly, food, *Artemia*, hatching and culture water were checked for contamination by plating on MA plates (n = 2). The plates were checked for absence of bacteria after incubation at 28 °C for 5 days. The experiment was discarded whenever the *Artemia* nauplii, water or food were found to be contaminated.

3.2.7. Statistical analysis

The data of nitrogen assimilation or enzyme digestive response from yeasts, bacteria and mixed yeast-bacteria diets in *Artemia* were first checked for homogeneity of variance and normality of distribution by the Levene's *F* test and P-P plot, respectively. As all data failed to meet these assumptions, the datasets were logarithmically and square root transformed. Statistical analysis was performed using Statistica 7.0 for Windows. All datasets were analyzed using one-way analysis of variance (ANOVA) followed by Tukey´s honestly significant difference (HSD) at 0.05 level of probability.

3.3. Results

3.3.1. Nitrogen assimilated by *Artemia* from bacterial strains HT3 and HT6

Assimilation of N from HT3 (Fig. 3.1) was gradually reduced when the proportion of bacteria in the mixed diets with yeast (either WT or mnn9) was reduced. However, there appeared to be differences in this trend between the different strains of yeast. When replacement of HT3 bacteria by mnn9 and WT was done, there was always more N assimilation in *Artemia* from HT3 when co-fed with WT than with mnn9. Replacement of HT3 up to 50% by yeast did not significantly change N assimilation compared to the control 1 ($p > 0.05$), except in case of 50% replacement with mnn9 (Fig. 3.1). Similarly, N assimilation derived from HT6 bacteria was gradually reduced when the proportion of this bacterial strain in the mixed diets with either type of yeast was reduced, but co-fed with HT6 there was no consistent difference between the two types of yeast (Fig. 3.1). A significant reduction ($p < 0.05$) of assimilation was already evident at 10% replacement with WT compared to the control. When the

[5] See 2.2.5

controls of the two bacterial strains HT3 and HT6 are compared, the latter allowed for more N assimilation in *Artemia* than the former.

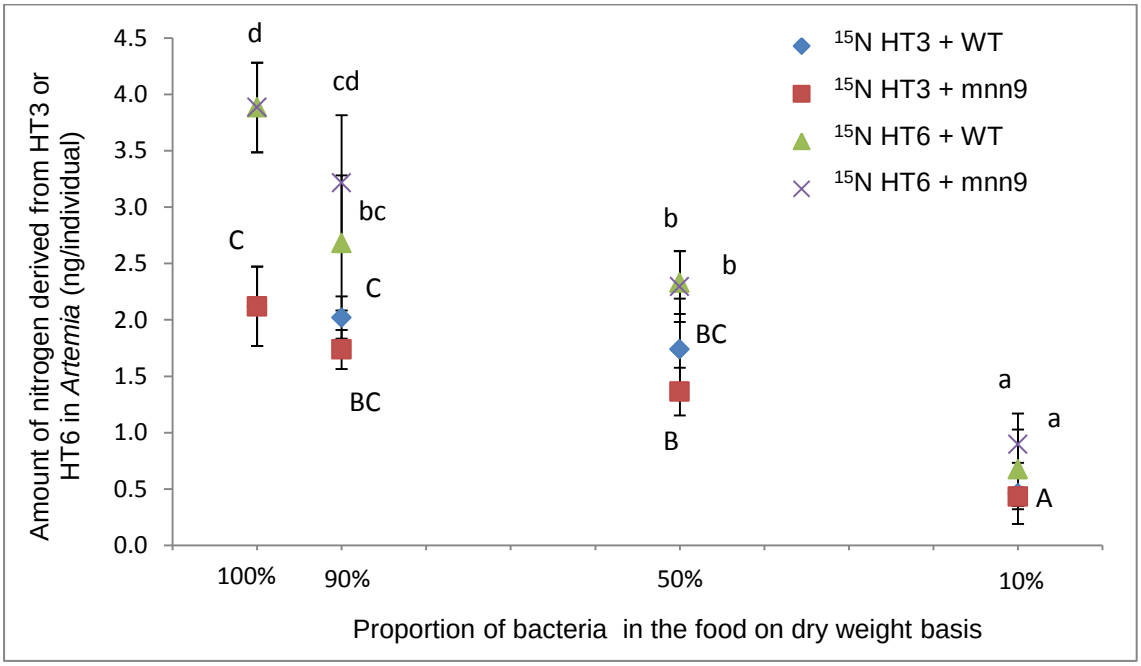

Figure 3.1: Effect of the presence of yeast on nitrogen assimilation from bacteria (ng/individual) in *Artemia*. The values are mean ± standard deviation (n = 3). Different letter superscripts of HT3 treatments and HT6 treatments indicate significant difference ($p < 0.05$) between the treatments. For the proportion of bacteria in the mixed diets, see Table 3.1.

3.3.2. Nitrogen assimilated by *Artemia* from yeast strains WT and mnn9

Nitrogen assimilation in *Artemia* from yeast strains WT and mnn9 (Fig. 3.2) was gradually reduced when the proportion of yeasts in the mixed diet with bacteria (either HT3 or HT6) was reduced. In wild type yeast (WT), a steady decline of N assimilation was evident when the proportion of WT in mixed diets was gradually replaced by bacteria. Except for 10% replacement of WT by HT6, N assimilation from WT in *Artemia* fed mixed diets was always significantly lower than the control ($p < 0.05$). For mnn9, in contrast, significantly lower N assimilation than the control was only obtained at replacement levels as high as 50% in case of HT3 and 90% in case of HT6. The combination with HT6 always produced higher N assimilation from both yeast types than the one with HT3. All treatments with the WT strain, including the control, resulted in much lower yeast-derived N assimilation than those with mnn9.

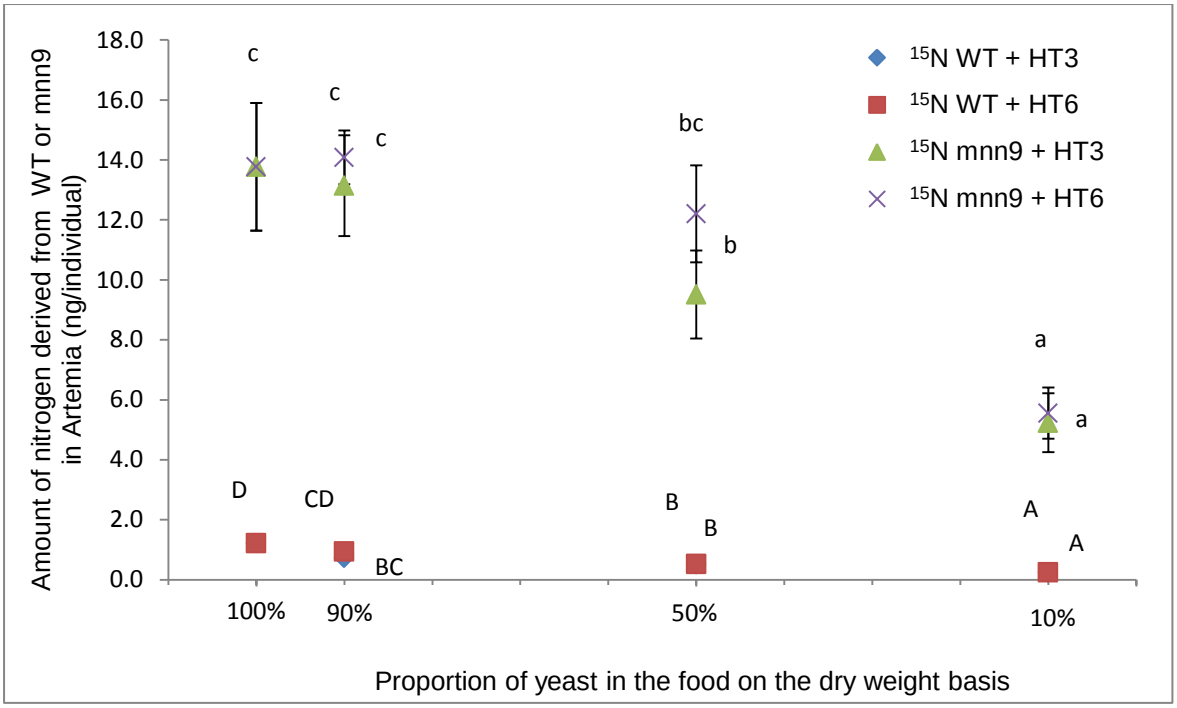

Figure 3.2: The effects of presence of bacteria on nitrogen assimilation from yeasts (ng/individual) in *Artemia*. The values are mean ± standard deviation (n = 3). Different letter superscripts of WT treatments and mnn9 treatments indicate significant difference of $p < 0.05$ between the treatments. For the proportion of yeast in the mixed diets, see Table 3.1.

3.3.3. Trypsin activity of *Artemia* fed mixed diets of yeasts and bacteria

No significant differences in trypsin-L activity were found among the treatments when replacing any of the two yeasts strains by either HT3 or HT6 bacteria (Fig. 3.3 A and 3.3 B, respectively), or when comparing the treatments with 100% yeast or 100% HT6 fed *Artemia*. In contrast, a significantly ($p < 0.05$) lower trypsin-L activity was found in the 100% HT3 control compared to all mixed diets and to other controls.

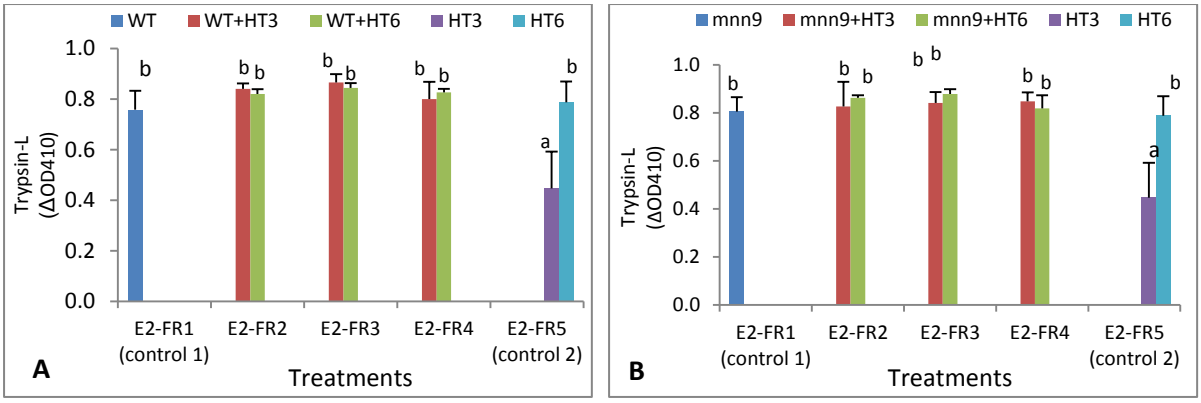

Figure 3.3: Effect of pure WT yeast and mixed WT yeast-bacteria diets (A) and of pure mnn9 yeast and mixed mnn9-bacteria diets (B) on trypsin-L activity in *Artemia* nauplii. Values are mean ± standard deviation (n = 3). Different letter superscripts within each figure indicate statistical significant difference of $p < 0.05$ between the treatments. For abbreviation of the treatments, see Table 3.1. For better comparison, control 2 (100% HT3 or HT 6) is shown on each graph.

3.4. Discussion

In our previous study we demonstrated bacterial isolates HT3 and HT6 associated with *Artemia* culture as being a suitable diet for *Artemia* nauplii (see chapter 2). In this study we determined the nitrogen assimilation from the food when these bacteria were offered to *Artemia* nauplii in a mixed diet together with wild type WT and mutant mnn9 yeast. Our data illustrate that HT6 provides for higher N assimilation in *Artemia* than HT3 when equal amounts (DW) of bacteria are offered, as evident when the respective FR1 (controls) are compared. This is a confirmation of our previous findings (chapter 2) where N assimilation from HT6 in *Artemia* was also higher than from HT3.

When both bacterial strains in the food are gradually replaced by yeast (WT or mnn9), the assimilation of N from bacteria in *Artemia* is also gradually decreasing, probably due to the reduced availability of bacteria when yeast is added. There are, however, differential effects on N assimilation depending on the replacing yeast strain and the available bacterial strain. When HT3 is present, replacement by WT always provides higher assimilation from bacteria, though not statistically significant, than replacement by mnn9 (Fig. 3.1). A first possible explanation for this difference between both yeast types could be that the presence of mnn9 as replacement provides more digestible food than replacement by WT (Marques et al., 2004b),

hence producing lower N assimilation from bacteria. Alternatively, WT may contribute for more exogenous enzymes than mnn9 such as proteolytic enzymes and alkaline phosphatase, leading to better utilization of bacteria and hence resulting in less reduction of N assimilation from HT3. According to Lara-Flores et al. (2003) the addition of yeast into a formulated diet for 3-week old fish Nile tilapia (*Oreochromis niloticus*, Linnaeus 1758) triggered alkaline phosphatase activity. High activity of alkaline phosphatase is believed to be related to increased nutrients absorption into enterocytes of fish (Gawlicka et al., 2000). Ozório et al. (2012) found the body weight of *O. niloticus* increasing 3 times at the end of the experiment (51 days), when yeast *S. cerevisiae* was added to formulated diets up to 20%. These data therefore suggest a strong potential of wild type yeast to assist in utilization of bacteria by *Artemia*. This action seems to be less prominent in mnn9 fed *Artemia*, co-fed with HT3, suggesting that mnn9, being a mutant, has not a similar positive effect on utilization of bacteria by *Artemia*.

However, these possibilities do not explain the interaction of HT6 and replacement by WT and mnn9, and its effect on N assimilation from the bacteria. In our experiment with HT6, mnn9 replacement instead of WT generally caused less reduction of N assimilation from HT6, though the differences were not significant. Higher N assimilation from HT6 than from HT3 may interact with mnn9 resulting into an increased N assimilation from bacteria (Fig. 3.1), though further studies are needed to prove or refute this hypothesis.

Our results further show that when yeast strains are replaced by HT3 and HT6, there is a gradual decline of N assimilation from yeast. This may be due to the reduced availability of yeast in the culture medium. Moreover, for both yeast types the N assimilation seems to be related to the bacterial strain used. With HT3, nitrogen assimilation from yeast is always less than with HT6 (Fig. 3.2). Literature data suggest that, other than being a dietary component, bacteria can also improve the availability of yeast nutrients to *Artemia*. The potential of bacteria as a source of digestive enzymes for *Artemia* has been reported previously (Intriago and Jones, 1993; Marques et al., 2004a). Significantly improved *Artemia* performance by the addition of bacteria as compared to axenic conditions has been reported by Douillet (1987) and Intriago and Jones (1993). Bacterial enzymes, such as N-acetyl-β-glucosaminidase (chitinolytic activity) and β-glucosidase (cellulolytic activity), β-

glucanase (glucalytic activity), and mannase were detected in selected bacterial strains (Fleet and Phaff, 1974; Araki et al., 1992; Rombaut et al., 1999) and their presence in the *Artemia* digestive tract may improve yeast digestibility, resulting in improved performance of *Artemia* (Marques et al., 2006a) as well as rotifers (Tinh et al., 2006).

Our study is the first to indicate that, when using the two yeast strains WT and mnn9 as sole diet, the latter results into a remarkably high N assimilation when it is fed to *Artemia* nauplii. Even though the assimilation of N has never been analyzed before, similar findings are reported elsewhere as exemplified by increased growth and survival of *Artemia* nauplii fed with mnn9 (Marques et al., 2004b). The mnn9 yeast mutant does not have a mannoprotein layer in the external cell wall (Marques et al., 2004b), rendering it more digestible as *Artemia* nauplii do not have mannase activity (Coutteau et al., 1990). Further, improved N assimilation in mnn9 may also be related to the higher nutrient content of mnn9, as indicated by its higher ash-free dry weight (Marques et al., 2004b; Soltanian et al., 2007). Gunasekara et al. (2011) reported that the length of the *Artemia* nauplius mid gut increases when *Artemia* is fed with mnn9 yeast compared to WT. The mid gut segment is more active in the absorbance of nutrients and the secretion of digestive fluids (Schrehardt, 1987). The experiment was performed for a short period (24 h) and as there were no obvious differences in *Artemia* length and survival among the treatments (results not shown) after 24 h, it was assumed that there was no difference in food consumption by *Artemia*, which might have affected N assimilation.

The enzyme assay of trypsin-L highlighted that whether mnn9 or WT is used, there is no significant difference in trypsin-L activity in *Artemia*. When HT6 is fed solely, the activity of trypsin-L is similar to that in *Artemia* fed solely WT or mnn9, suggesting that, within the time duration of our experiment, WT, mnn9 and HT6 stimulate similar levels of trypsin-L activity, be it fed solely or in combination. Only *Artemia* fed solely HT3 showed significantly lower levels of trypsin-L than all other feeding treatments. This effect is not present in treatments where HT3 is fed in combination with yeast. This finding is in agreement with our previous study where the presence of HT3 lead to lower N assimilation from microalgae in *Artemia* when compared to the presence of HT6 (see chapter 2). Similar negative effects of bacteria on trypsin-L activity in *Artemia* have been reported previously (Rojas-García et al., 2008). The negative

effects of HT3 on trypsin-L activity and its compensation by the yeast strains show that the yeast strains tested here can modulate digestive functions of *Artemia* in a favorable manner.

In conclusion, bacteria and yeast not only may play an important nutritional role in *Artemia* when fed as sole diet, but their joint presence in *Artemia* culture may also improve nitrogen assimilation in *Artemia*.

Chapter 4

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

Modified from:

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Chapter 4: Bacteria contribute to *Artemia* nutrition in algae-limited conditions: a laboratory study

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.

4.1. 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, are a suitable substitute for the zooplankton that is the natural food of 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), tiger shrimp (*Penaeus monodon*) 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, biofloc is a protein-rich food source available *in situ* 24 h per day for consumers (Emerenciano et al., 2013). Therefore, promoting bacterial growth in aquaculture systems clearly reduced the need for artificial food (Avnimelech, 1999; Burford et al., 2004; Hari et al., 2004; Crab et al., 2009b) as compared to the culture without promoting bacterial growth.

Artemia pond production of cysts and biomass is a profitable activity in solar saltworks in the Mekong Delta, Vietnam (Baert et al., 1997; Anh, 2009). 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 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; Hari et al., 2006; Crab et al.,

2009b; 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 ratio 50 was chosen as this is far above the range 10-20 described in literature. Moreover, the size of bioflocs depends on the C/N ratio; a smaller size of flocs is obtained at C/N ratio 4 as compared to C/N ratio 20 and 100 (Ye et al., 2011b). 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 addition of ^{15}N-nitrogen into the *Artemia* cultures to label the bacteria (Burford et al., 2004; Avnimelech and Kochba, 2009) 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*.

4.2. Materials and methods

4.2.1. Experimental design

Artemia was cultured over a period of 15 days 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. 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 4.1). 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 4.1: 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 Day 5-14
	Day 1-4	Day 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*	-	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

*: 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:

N needed (mg) per day = algae N content in SF (mg) - algae N content in LF (mg)

The carbon sources and inorganic nitrogen were daily adjusted according to the feeding regime (Table 4.2). ^{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.

4.2.2. 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 a pH change over a period of 6 h with continuous illumination (± 41 µE/m^2s) 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 4.2).

Table 4.2: 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

4.2.3. *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 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 $NaHCO_3$ 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.

4.2.4. Data collection and sample analysis

4.2.4.1. 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* were filled completely with cellulose.

4.2.4.2. 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., 2004b), and by conversion to real length using the software *Artemia* 1.0® (courtesy of Marnix Van Damme).

4.2.4.3. 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:

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

4.2.4.4. 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 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 ^{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 (‰).

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

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

4.2.4.5. 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 20:2n-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.

4.2.5. 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 the Levene's *F* test and 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. Three-way analysis of variance (ANOVA) was used to test the effect of feeding regime, carbohydrate source and C/N ratio, and the interaction among these factors, on survival, individual length and biomass production of *Artemia*. Additionally, Tukey´s honestly significant difference (HSD) post-hoc test at 0.05 probability level was employed. One-way ANOVA was performed for fatty acid levels and ^{15}N accumulation in the *Artemia* tissue.

4.3. Results

4.3.1. *Artemia* performance

The feeding regime and carbon source significantly affected survival of *Artemia*, and interaction between these variables was significant ($p < 0.05$) (Table 4.3). For survival, there was also significant interaction between feeding regime and C/N ratio. The growth of *Artemia* was significantly affected by feeding regime, carbon source and C/N ratio; there was significant interaction between feeding regime and carbon source and between the three variables ($p < 0.05$).

Finally, there was a significant effect on biomass production by feeding regime and C/N ratio, and significant interaction between both these variables ($p < 0.05$) (Table 4.3).

Table 4.3: Probability levels of three-way analysis of variance (with feeding regime, carbon source and C/N ratio 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)	0.002*	0.000*	0.000*
Carbon source (CS)	0.048*	0.046*	0.124
C/N ratio	0.164	0.000*	0.000*
FR x CS	0.028*	0.585	0.883
FR x C/N ratio	0.003*	0.000*	0.000*
CS x C/N ratio	0.139	0.361	0.117
FR x CS x C/N ratio	0.270	0.001*	0.148

Survival in SF-fed *Artemia* was significantly higher than in LF-fed *Artemia* ($p < 0.05$) (Table 4.4). 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 ($p < 0.05$).

Table 4.4 : 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 4.1.

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

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, and in case of sucrose at both C/N ratios the increase was significant ($p < 0.05$).

4.3.2. Fatty acid composition of food and *Artemia*

4.3.2.1. 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 4.5).

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 4.5). 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, 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, 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 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 the control, but this decrease was not significant ($p > 0.05$), except for ST50 (Table 4.5). 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 4.5).

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 4.5: 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. Fatty acids, discussed in the text, are highlighted in grey. 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 4.1. 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	-

Table 4.5, continued

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
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	-

Table 4.5, continued

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
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}

4.3.3. 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. 4.1). 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 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 ($p < 0.05$).

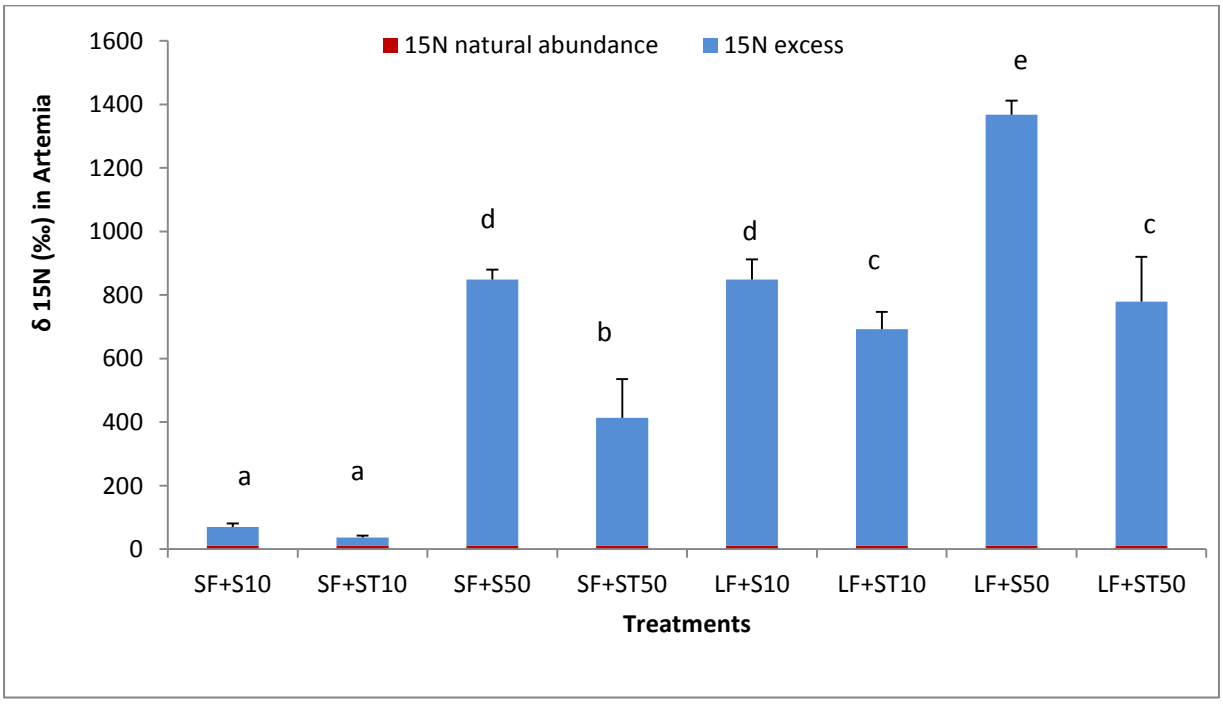

Figure 4.1: Nitrogen accumulation in *Artemia* biomass fed two different feeding regimes and different culture conditions stimulating bacterial growth. Values are mean ± standard deviation (n = 3). Indices a, b, c and d are indicating homogenous subsets ($p < 0.05$). For abbreviations of treatments, see Table 4.1.

4.4. 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; Hari et al., 2004; Crab

et al., 2009b; 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 (Intriago and Jones, 1993; Erasmus et al., 1997).

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* was 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 4.5), 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 (Burford et al., 2004; Avnimelech and Kochba, 2009). 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 can not 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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Chapter 5

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

Chapter 5: Contribution of bacteria to *Artemia* nutrition in conditions of degressive algal feeding

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 ^{15}N 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.

5.1. 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., 2009a; Nootong et al., 2011).

In our previous study (Toi et al., 2013) we 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.

5.2. Materials and methods

5.2.1. Experimental design

Based on the results of biomass production in our previous study, and to avoid the lower culture output found in the treatments with starch addition and those with C/N 50 (chapter 4), the addition of inorganic nitrogen was eliminated and only C/N ratio 10 was applied in this study, using only sucrose from the 2nd day after hatching (DAH2) onwards. *Artemia* were reared on four different microalgae *Tetraselmis* sp. rations under laboratory conditions during 15 days (Table 5.1). 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 5.1), 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*, ^{15}N $NaNO_3$ 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 5.1: 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
1. SF1 (control 1)	standard feeding	-
2. SF1+S10	standard feeding	sucrose
3. SF1/2 (control 2)	half of standard feeding	-
4. SF1/2+S10	half of standard feeding	sucrose
5. SF1/3 (control 3)	one third of standard feeding	-
6. SF1/3+S10	one third of standard feeding	sucrose
7. SF1/4 (control 4)	one fourth of standard feeding	-
8. SF1/4+S10	one fourth of standard feeding	sucrose

5.2.2. *Artemia* hatching and culture procedures[6]

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 $NaHCO_3$ 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.

[6] See 4.2.3

5.2.3. Food preparation[7]

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/m^2s) 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 5.2)

Table 5.2: 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

5.2.4. Data collection and sample analysis

5.2.4.1. Pre-sampling treatment[8]

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

[7] See 4.2.2
[8] See 4.2.4.1

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.

5.2.4.2. Growth of *Artemia*[9]

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)

5.2.4.3. Survival and total biomass production[10]

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

5.2.4.4. Nitrogen accumulation in *Artemia*[11]

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

[9] See 4.2.4.2
[10] See 4.2.4.3
[11] See 4.2.4.4

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 ^{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 (‰).

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

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

5.2.4.5. Fatty acids composition of *Artemia* and feed sources[12]

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 20:2n-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

[12] See 4.2.4.5

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.

5.2.5. 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 treatment$_i$ – survival or TBP of control$_i$)/ survival or TBP of control$_i$

Where i=1-4

5.2.6. Statistical analysis

The data of biomass production, fatty acid levels and the values of ^{15}N 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 ^{15}N accumulation in the *Artemia* tissue.

5.3. Results

5.3.1. *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 5.3).

Table 5.3: 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 5.4).

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 5.4: 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 5.1.

Treatment code	Survival (%)	IL (mm)	TBP (g/L)	RSI (%)	RTBPI (%)
1. SF1 (control 1)	36.4 ± 12.7^{ab}	7.0 ± 1.6^{bc}	2.8 ± 0.6^{cd}	-	-
2. SF1+S10	62.4 ± 25.8^{bc}	7.9 ± 1.7^{c}	4.6 ± 1.7^{d}	84.3 ± 63.0^{ab}	60.2 ± 35.1^{a}
3. SF1/2 (control 2)	41.1 ± 13.4^{abc}	6.7 ± 1.1^{abc}	2.1 ± 0.2^{abc}	-	-
4. 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 ± 32.7^{a}
5. SF1/3 (control 3)	22.7 ± 4.1^{a}	7.2 ± 1.4^{bc}	1.2 ± 0.3^{ab}	-	-
6. 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}
7. SF1/4 (control 4)	14.4 ± 8.1^{a}	6.4 ± 1.3^{ab}	0.6 ± 0.3^{a}	-	-
8. SF1/4+S10	42.0 ± 7.6^{abc}	6.1 ± 1.3^{ab}	1.4 ± 0.3^{ab}	192.9 ± 53.3^{b}	146.4 ± 99.0^{a}

5.3.2. Fatty acids composition of feed and *Artemia*

5.3.2.1. 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).

5.3.2.2. 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 5.5). 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 5.5: 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 5.1.

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 ± 0.1^{a}	0.5 ± 0.1^{ab}	0.2 ± 0.1^{a}	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

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
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 ± 1.7^{abc}	6.5 ± 0.6^{a}	8.1 ± 0.6^{bcd}	6.7 ± 0.9^{ab}
18:4n-3	11.6 ± 0.5	0.1	3.6 ± 0.7^{c}	3.8 ± 0.5^{c}	2.6 ± 0.0^{abc}	3.0 ± 0.3^{abc}	2.5 ± 0.8^{ab}	2.5 ± 0.2^{a}	2.6 ± 0.3^{abc}	2.5 ± 0.1^{a}
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 ± 0.2^{ab}
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	-	-	-	-	-	-	-	-	-	-

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
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 ± 1.9^{bc}	26.0 ± 2.1^{c}	21.1 ± 0.2^{abc}	23.6 ± 1.9^{bc}	20.3 ± 1.2^{ab}	25.6 ± 2.5^{c}	19.5 ± 0.2^{a}	23.7 ± 0.6^{c}
PUFA	57.1 ± 2.6	2.7	27.5 ± 1.2^{b}	26.4 ± 1.9^{ab}	23.9 ± 1.2^{ab}	23.4 ± 0.9^{ab}	21.8 ± 2.1^{ab}	19.7 ± 1.3^{a}	23.2 ± 1.5^{ab}	20.2 ± 0.6^{ab}
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

5.3.3. Nitrogen derived from heterotrophic bacteria

The natural ^{15}N abundance in *Artemia* was as low as 10‰ (Fig. 5.1).The excess of ^{15}N 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 ($p < 0.05$).

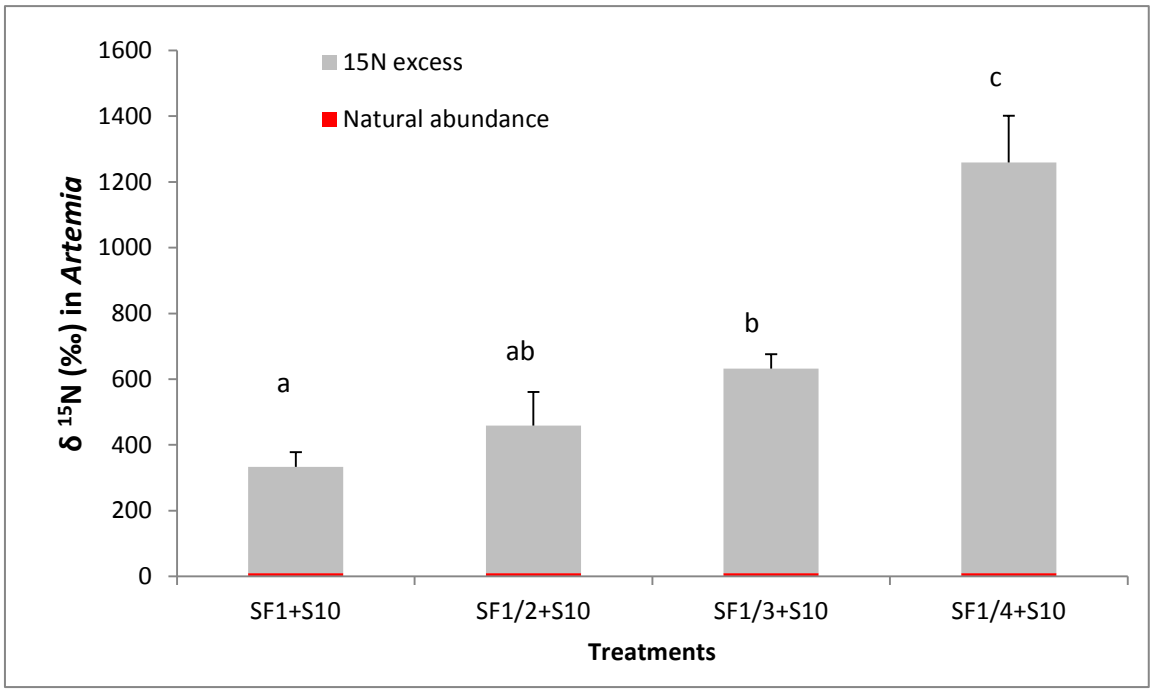

Figure 5.1: ^{15}N nitrogen accumulation in *Artemia*. The values are mean ± standard deviation (n = 3). Indices a, b, c, and d are indicating homogenous subsets ($p < 0.05$). For abbreviation of the treatments, see Table 5.1.

5.4. 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 our previous findings (see 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 5.5), 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 our 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 ^{15}N 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 ^{15}N 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 ^{15}N versus MUFA accumulation in *Artemia*, there was dramatically gain of ^{15}N 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.

Chapter 6

General discussion

Chapter 6: General discussion

6.1. Introduction

The classical operation procedure of *Artemia* pond culture in the Mekong delta of southern Vietnam, *Artemia* is offered plenty of food sources such as microalgae, inert diets (rice bran, soybean meal) and chicken manure (Baert et al., 1997; Anh, 2009). In recent years, biofloc-based techniques have been applied to *Artemia* ponds as well (Ronald et al., 2013; Sui et al., 2013). However it is not clear to what extent bacterial biomass has a nutritional contribution to the *Artemia* diet. Assessment of the nutritional contribution of bacteria is difficult to perform in *Artemia* pond culture systems, where *Artemia* gets plenty of food sources. Moreover, field factors such as uncontrolled variation of temperature, salinity, dissolved oxygen etc, interfere with the experimental set-up (Ronald et al., 2013).

Therefore, in the present study, the experiments aimed at verifying the dietary role of bacteria in the *Artemia* diet under controlled laboratory conditions. Firstly the suitability of specific heterotrophic bacteria to sustain *Artemia* growth and culture over a 6-day culture period was demonstrated in gnotobiotic culture conditions. Subsequently food assimilation in *Artemia* nauplii was investigated, when *Artemia* were fed bacteria in gnotobiotic culture conditions, either as mono-diet or in combination with other microorganisms such as microalgae and yeasts. Finally, we examined the suitability of bacterial biomass as a substitute for microalgae in prolonged conventional xenic *Artemia* cultures, where microbial floc development was stimulated in conditions of reduced microalgae supply. Aiming at the conversion of nitrogen waste from *Artemia* into bacterial cells, which in turn can be used as food source for *Artemia*, ^{15}N isotope was used to label bacteria in the xenic cultures. Then the ^{15}N accumulation in the *Artemia* tissue was measured. This technique was also used to study nitrogen assimilation in *Artemia* in the presence of bacteria, yeast and microalgae in the gnotobiotic cultures.

6.2. The dietary role of associated bacteria in *Artemia*, as demonstrated in gnotobiotic culture conditions

The first step of this investigation aimed to exemplify whether heterotrophic bacteria, which can grow on an organic carbon source and inorganic nitrogen source, may sustain growth and survival in *Artemia*. Nine bacterial strains (HT1-HT9), originating from a well-performing *Artemia* laboratory culture, where *Artemia* was reared with water from a marine shrimp *Litopenaeus vannamei* recirculation culture system and WT yeast, were screened in this study (chapter 2). It was assumed that this water would contain heterotrophic bacteria, which can grow on the carbohydrate sources supplied through the uneaten food and on the metabolic nitrogen wastes from the shrimp.

Making use of the gnotobiotic approach, it was hypothesized that *Artemia* may get nutrients from the bacteria, when reared under suboptimal feeding conditions, namely with only bacteria as the main food source. The reference bacterial strain LVS3 (*Aeromonas hydrophila*), which also originated from well-performing *Artemia* cultures, with proven positive effects on *Artemia* (Verschuere et al., 1999; Marques et al., 2005), was also used alongside to compare the effects of isolates on *Artemia* survival and growth.

In our observation, *Artemia* could survive until day 6 on some isolates such as HT3, HT6, HT7 and HT8, while starved *Artemia* could not survive to day 6. According to the investigation conducted by Marques et al. (2005), starved *Artemia* nauplii could not survive more than 4 days in axenic culture conditions. *Artemia* nauplii in our observation may thus have utilized nutrients derived from the bacterial cells such as proteins and lipids (Brown et al., 1996), which are the main nutrients required for the growth of the early stages of *Artemia* (Marques et al., 2005). Among nine isolates, the bacterial strains HT3 (*Tamlana* sp. ZJU HZ22) and HT6 (*Bacillus subtilis* 168) showed a higher positive effect on *Artemia* performance based on survival and individual body length compared to the other isolates and this positive effect was even higher or identical to the reference LVS3. By using these experimental culture conditions, Verschuere et al. (1999) demonstrated that some associated bacteria such as LVS2 (*Bacillus* sp.), LVS3 and LVS8 had positive effects on *Artemia* performance. Later the effect of some of these isolates on *Artemia* performance has

been confirmed by Marques et al. (2005). The findings from Verschuere et al. (1999) and our findings indicate that associated bacteria which originated, in our case, from a recirculation shrimp culture, may serve as a direct nutritional source to *Artemia*. However the nutritional value of bacteria is species-specific, and may even be strain-specific. Thanks to its filtering feeding behavior, brine shrimp can thus be a potential species for extractive aquaculture, aiming to reduce nutrient loads in shrimp effluents by promoting bacterial growth which in turn can be the food source for filter-feeders.

In a subsequent step (chapter 2 and 3), we evaluated the N assimilation from different types of microorganisms, such as microalgae and yeasts, having differences in nutritional quality, when they were co-fed with the selected bacteria strains HT3 and HT6. Live cells of these two bacterial strains were co-fed with *Dunaliella tertiolecta* DT 19/6B or DT 19/27. The results show that when offered mono-diets, the N assimilation from both bacterial strains (HT3 and HT6) was more or less equal to the N assimilation from the poorly digestible WT yeast, but lower than from mnn9 and from both strains of microalgae. Moreover, N assimilation from DT 19/27 was higher than from mnn9 but lower than from DT 19/6B. This is in agreement with the data on growth performance of *Artemia* under gnotobiotic conditions when these cells were offered as single food items (Marques et al., 2004). Nutritional value might be a primary factor influencing N assimilation.

Yet, not only the nutritional content is important, but also the particle size, as it can affect grazing efficiency. The ash-free dry weight of mnn9 yeast (around 29.5 mg/10^9 cells) and DT 19/6B (around 90.4 mg/10^9 cells) is higher than for WT yeast (around 15.1 mg/10^9 cells) and DT 19/27 (around 75.3/10^9 cells), respectively (Marques et al., 2005). The clearance rate of *Artemia* fed particles of bacterial size (about 0.5 µm) is 69 times less than when fed particles of algal size (about 12 µm) (Moore and Jaeckle, 2010). The size of *D. tertiolecta* and baker´s yeast *S. cerevisiae* cells is around 15.0 µm (da Silva Gorgônio et al., 2013) and around 5.5 µm (Portell et al., 2010), respectively, which is substantially larger than bacterial cells (but as can be deduced from the ash-free dry weight, mnn9 yeast cells are much larger than WT cells). In terms of size, yeast and microalgae may thus be better grazed by *Artemia* nauplii as compared to bacteria (even if equal biomass is offered).

The grazing efficiency (clearance rate) might also be influenced by the simultaneous presence of particles of different size. In the presence of both bacteria (size 0.5 µm)

and *Tetraselmi*s sp. (8.0 µm), the clearance rate of *Artemia* for the *Tetraselmis* cells was around 5 times higher than for the bacterial cells (Makridis and Vadstein, 1999), in case the density of bacterial particles and *Tetraselmi*s sp. in the mixed solution was approximately 2 x 10^7 cells/mL and, 5 x 10^4 cells/mL, respectively. When offered as single diet, however (the concentration of bacteria and microalgae was around 6 x 10^6 cells/mL and 5 x 10^3 cells/mL, respectively), this was 69 times higher for the microalgae (Moore and Jaeckle, 2010). These literature data seem to indicate that the clearance rate of a particle of a particular size is strongly influenced by the overall particle composition, pointing in the direction of reduced algal clearance when bacteria are around. In future research, this possibility should be taken into consideration when studying N assimilation by *Artemia*. A technical approach to this problem could be to offer algal cells either in the presence of bacteria or in the presence of inert particles of equal size, as to avoid that N assimilation would be blurred by difference in clearance rate (although the latter should also be closely monitored).

The presence of multiple feed sources may be also increase digestion and assimilation of food in *Artemia*. The mixed diet may trigger the digestion process by stimulating enzymatic activity in the *Artemia* gut, and the mixed diet may also provide digestive enzymes from the lysed cells. Together with enzymes of *Artemia*, they may degrade bacteria, making them easier to absorb by *Artemia*, resulting in an increase of N assimilation. More detailed study on the clearance rate of food particles (either as mono-diets or multiple diets), and digestion of food in the presence of bacteria and yeast or microalgae will be needed to gain insight on the influence of bacteria on N assimilation in *Artemia*.

When co-feeding with bacteria, the N assimilation from microalgae was slightly increased or nearly identical as compared to *Artemia* fed solely microalgae, although the concentration of microalgae was reduced with 10% and 50% in the mixed diets. The reduced supply of microalgae should result in a reduced N assimilation from this feed source, though this should be confirmed by similar experiments using a mono-diet of algae (or yeast or bacteria) at 10% and 50% of the standard feeding ration. In contrast to microalgae, the N assimilation from yeast was gradually reduced when the proportion of yeast was gradually replaced by the bacterial strains.

Moreover, the N assimilation from both yeast and microalgae strains in *Artemia* seems related to the bacteria strains used in the mixes. In the presence of HT3, the N assimilation from yeast and microalgae was less than in the presence of HT6. Similarly, the N assimilation from bacteria was related to the strain of microalgae and yeast used in the mixed diets. Co-feeding with DT 19/6B always induced better N assimilation from bacteria than with DT 19/27. The presence of DT 19/6B increased N assimilation from both strains of bacteria, although the proportion of bacteria was reduced with up to 50%. The N assimilation from HT6 bacteria even nearly doubled when 10% of HT6 was replaced by DT 19/6B in comparison to solely HT6 fed *Artemia*. In the case of co-feeding with yeast, the presence of WT induced better N assimilation from HT3 than mnn9.

The improved N assimilation could also be explained by modulation of trypsin expression. Trypsin, a key enzyme for protein digestion, is the digestive enzyme that facilitates digestion and assimilation of different food items (Lovett and Felder, 1989; Savoie et al., 2011). Trypsin plays an important role in the digestion of proteins necessary for the lecithotrophic development in cysts and the early stages of *Artemia* (Stabili et al., 1999). The *Artemia* nauplius starts food uptake in the second instar stage (Van Stappen, 1996), and larval digestive enzyme activities respond to food conditions (Samain et al., 1985). In our current study, trypsin activity in *Artemia* was measured to evaluate the digestive response in *Artemia* in mono-diets as compared to mixed diets. In our observation the trypsin activity was nearly identical between *Artemia* fed mono-diets and mixed diets (except for a significantly lower trypsin activity in HT3 fed *Artemia*), illustrating that trypsin activity is not modulated under the described conditions and that other unknown mechanisms may be involved in the improved N assimilation in the joint presence of yeast and bacteria or microalgae and bacteria.

More studies on different digestive enzymes activities and on the exact mechanisms of N assimilation in *Artemia* under the described experimental conditions will be needed to gain more insight into this process.

6.3. Implementation of the biofloc technique in *Artemia* culture

6.3.1. Experimental design

In chapters 4 and 5, the effect of promoting heterotrophic bacterial growth on *Artemia* performance was evaluated. Fatty acids signature and ^{15}N stable isotope were used as tools to quantify the contribution of bacteria to the *Artemia* diet when given different microalgal feeding rations. Non-viable cells of *Tetraselmis* sp. were chosen as a reference food for *Artemia*, assuming that ^{15}N assimilation was not done by non-viable cells in light conditions (see chapter 2 and 3). Good growth of *Artemia* on *Tetraselmis* has been documented before (García-Ulloa Gómez et al., 1999; Thinh et al., 1999; Godínez et al., 2004; Marques et al., 2004a; Seixas et al., 2009), with growth on this algal species being even better than on *D. tertiolecta* (Marques et al., 2004a).

Two different carbohydrate sources, sucrose (disaccharide) and starch (polysaccharide) were used to alter the apparent C/N ratio to 10 and 50. C/N ratio in nature can vary strongly, basically from around 3 (pure protein) to more than 200, e.g. in the mangrove leaves (Woitchik et al., 1997). With a yield coefficient of around 50% in microbial growth (50% of the substrate is transformed into microbial biomass) and a C/N ratio of microbial biomass of roughly 5, one can say that starting from C/N ratio 10 and higher, in practice somewhere in between 10 and 20, all nitrogen becomes fixed in microbial biomass. Hence in our experiments, as we did not know the kinetics of N release and a wanted to ensure a high C/N ratio at all times, we went for a very high C/N ratio (50), knowing that this is slightly exaggerated, in relation to the need of C to fix all N. Two different algal feeding regimes, namely standard (SF) and low (LF; the latter being 1/4 of SF) were applied to culture *Artemia*. For each algal feeding regime, two different bacterial feeding regimes were established resulting in an apparent C/N ratio of 10 and 50. The results showed that the addition of the two carbohydrate sources increased survival, body length and biomass production of *Artemia*. This was the case in all LF regime treatments and for the SF treatment when carbon was supplied at an apparent C/N ratio of 10. Under the SF regime, the addition of carbohydrate at C/N ratio 50 resulted in a lower *Artemia* survival, generally a smaller body length and less biomass production.

These results indicate that operational parameters might have a very strong influence on biofloc characteristics on the one hand and on nutritional value on the other hand.

6.3.2. Operational parameters influencing biofloc production

In this study three operational parameters, namely algal feeding regime, C source and apparent C/N ratio may have influenced biofloc formation (see Fig. 6.1). The algal feeding regime in combination with the applied C/N ratios in this study will have influenced indirectly the “organic loading rate” of the bioflocs. The latter is known to influence biofloc composition in relation to the growth of floc-forming or filamentous microorganisms (De Schryver et al., 2008). Hence it appears from this study that it might be of interest to quantify and control this organic loading rate in biofloc-based *Artemia* pond production. For this purpose it will be necessary to monitor carefully the amount of bioflocs available in the pond, which could be done by measuring the amount of suspended solids (SS in g/L) or volatile suspended solids (VSS in g/L) on the one hand and by controling C input on the other hand. This was not done in this study, as the focus was on establishing different apparent C/N ratios. Finally the mixing intensity appears to be an important parameter influencing floc structure and size. These parameters were not considered in this study but they may be very important to investigate in future field studies in biofloc-based *Artemia* production, as they may influence bioflocs but also directly *Artemia*. The direct physical influence on *Artemia* may limit the degree of mixing that can be applied.

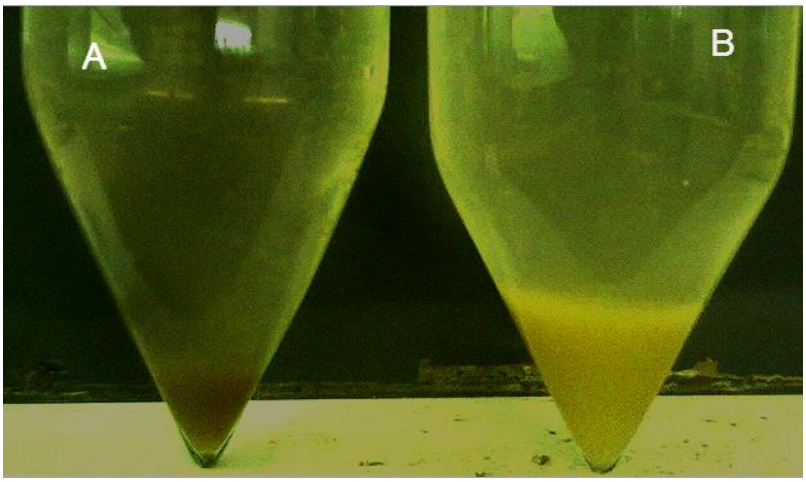

Figure 6.1: Bioflocs produced on sucrose at C/N ratio 10 (A) and C/N ratio 50 (B) under a standard algal feeding regime in *Artemia* cultures (bioflocs after 30 min of settlement)

6.3.3. *Artemia* food uptake in relation to floc density and size

The performance of *Artemia* may be also related to the availability/density/size of food. A massive growth of bacteria was found in the cultures where *Artemia* received the standard algal feeding regime in combination with a C supply at C/N ratio 50 (see Fig. 6.1). The massive growth of bacteria together with microalgae in the standard feeding regime may lead to overfeeding for *Artemia* and increase water viscosity, which may result in retarded growth (Nimura, 1980) (see chapter 4).

The formation of microbial flocs is affected by various environmental water parameters (De Schryver et al., 2008): e.g. temperature, pH, salinity, dissolved oxygen etc. In our study, the *Artemia* was reared in identical standard culture conditions for all treatments. So floc size might have been modulated by the type of carbohydrate and by C/N ratios. Ye et al. (2011b) reported that the size of microbial flocs was related to the C/N ratio. In activated sludge systems, a smaller size was observed at C/N ratio 4 as compared to C/N ratio's of 20 and 100. But in the activated sludge system different carbohydrate sources such as starch, glucose and sodium acetate, did not seem to influence floc size (Ye et al., 2011a). Size of flocs in the culture where *Artemia* were offered the standard feeding regime at C/N ratio 50, may have exceeded the uptake size of *Artemia* (see chapter 1).

It is clear that floc quantity and floc size are two important parameters to be controlled in biofloc-based *Artemia* ponds. Although very little studies have been done under field conditions, it appears that proper control of some operational parameters (see Table 6.1 and own results under laboratory conditions) can help to offer sufficient flocs of the right size to *Artemia*.

6.3.4. Digestion and assimilation of bioflocs by *Artemia*

The results of ^{15}N accumulation and fatty acid profiles demonstrated that *Artemia* is capable of microbial flocs digestion and assimilation. The extent of bacterial biomass digestion and assimilation by *Artemia* depends on a variety of parameters. Firstly the ratio between microalgae and bacteria being supplied seems to be important. As compared to the control without carbohydrate addition (and hence bacterial growth), fatty acid levels, particularly of 16:1n-7 and 18:1n-7 and ^{15}N showed a higher increase in the *Artemia* biomass under a low algal feeding regime as compared to

the high algal feeding regime. For instance at an apparent C/N ratio of 10, the 16:1n-7 level in *Artemia* under the low algal feeding regime (LF) (¼ of the standard feeding regime) was 2 to 3.6 times higher than in *Artemia* at standard feeding (SF) regime, while ^{15}N accumulation was 14 to 26 times higher (chapter 4). So bacterial assimilation seems to increase when the proportion of algae in the diet decreases. This might indicate preferential digestion and assimilation of algae.

There is however a technical limitation in using fatty acid markers for monitoring microbial biomass assimilation. In most organisms, some fatty acids can be elongated, e.g. 16:1n-7 can be elongated to yield stearic acid (18:0) and vaccenic acid (18:1n-7) (Green et al., 2010), and 16:1n-5 is elongated to 18:1n-7 in bacteria-fed *Artemia* (Intriago and Jones, 1993). Thus 16:1n-7 and 18:1n-7 could be used to follow the fate of bacterial nutrients in consumers, but not for accurate quantification of the bacterial nutrients transferred to *Artemia* in our studies. The combination of ^{15}N and fatty acid analyses however has proven to be a helpful approach to illustrate to what extent bacteria may contribute to the *Artemia* diets.

Microbial biomass composition might be another factor determining assimilation. Crab (2010) observed that the different carbon sources stimulated specific bacteria, influencing the microbial composition and community organization of bioflocs and thereby also their nutritional properties. Bioflocs grown on glucose (monosaccharide) have a protein content (around 40%) and crude lipid content (around 41%) which was higher than bioflocs grown on starch (polysaccharide) where protein content was around 21% and crude lipid content was around 17%. The different C/N ratios may affect nutritional properties of microbial flocs. The protein content of activated sludge flocs (around 40 mg/g SS), grown at C/N ratio 4, was higher than the protein content of activated sludge flocs grown at a C/N ratio 20 and 100 (around 15 mg/g SS) (Ye et al., 2011b). The difference of protein or lipid content in flocs may be due to differences in microbial composition of the flocs. The results in our study (chapter 4) showed that two different carbohydrate sources did not produce significant differences in *Artemia* performance, but at C/N ratio 10 *Artemia* performed slightly better when sucrose was added in comparison with potato starch. In future studies detailed microbial flocs composition analysis might help to determine how composition determines digestion and assimilation of flocs.

Microbial flocs are not only a supplementary food source, but microbial flocs can also be a source of digestive enzymes of some target aquatic organisms. Becerra-Dórame et al. (2012) and Xu and Pan (2012) reported increased activity of digestive enzymes such as trypsin and amylase in postlarval shrimp *Litopenaeus vannamei* in the culture where the growth of microbial flocs was stimulated. Inclusion of microbial flocs as a dietary ingredient of juvenile shrimp *Penaeus monodon* was found to increase the digestive enzyme activities in the gut and hepatopancreas (Anand et al., 2014). The increase of digestive enzyme activities may have improved the digestion and nutrient assimilation which may have contributed to growth. In the case of *Artemia*, the activity of digestive enzymes may similarly increase in the presence of microbial flocs. However in this study digestive enzyme activity (trypsin) in *Artemia* was not modulated in the cultures where the growth of microbial flocs was stimulated. Therefore, investigation on the increase of digestive enzyme activities (focusing on more digestive enzymes) in the presence of microbial flocs is required to gain insight in their contribution to flocs assimilation by *Artemia*.

The results of *Artemia* biomass production from our study showed that up to 67% reduction of microalgae could be compensated by bacteria (chapter 5), whereas this was not the case for 75% reduction of microalgae (chapter 4 and chapter 5). Martin-Creuzburg (2013) found that the somatic growth of *Daphnia magna* was not affected when 50% of algae *Scenedesmus obliquus* in the diet were replaced by bacteria *Flavobacterium* sp. This suggests that the microbial mass lacks some nutrients required for growth. According to Phillips (1984) most bacteria lack both polyunsaturated fatty acids (PUFA) and sterols, whereas these are available in microalgae. It is known that certain PUFA are essential in the diet of marine organisms. For example eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) have been shown to be essential for seabream *Sparus aurata* (Rodríguez et al., 1998), marine shrimp *Penaeus monodon* (Glencross et al., 2002) and *Penaeus japonicus* (Kontara et al., 1997), while *Artemia* requires linoleic (18:2n-6) and linolenic acid (18:3n-3) for growth (Intriago and Jones, 1993). At the moment the quantity of both linoleic and linolenic acid needed for the growth of *Artemia* still remains unknown. The results of fatty analysis in our study (chapter 5) showed that the bacterial mass grown on sucrose contained lower linoleic and linolenic acid

levels than the *Tetraselmis* concentrate, which may be linked to the lower *Artemia* performance at 75% algae substitution.

On the other hand, like all arthropods, crustaceans are incapable of synthesizing sterols, which they must obtain from their diets (Goad, 1981). According to Klein Breteler et al. (1999) sterols are indispensible for several vital functions such as maintenance of cellular architecture, membrane functioning, growth and reproduction. Elert et al. (2003) reported that cholesterol addition (2.5 mg/L) improved the growth of bacteria-fed *Daphnia*. But the addition of cholesterol at 100 µg/L and 500 µg/L did not improve the growth and survival of bacteria-fed *Artemia* (Intriago and Jones, 1993), may be because the dosage of cholesterol supplement was lower than the requirements to maintain *Artemia* growth. Sterol is naturally available in marine microalgae (Ponomarenko et al., 2004), so the presence of microalgae may compensate for the cholesterol-deficient bacteria and result in good *Artemia* growth, as shown in our study.

6.4. Conclusions and perspectives for future study

From the results of this study the following conclusions can be drawn: gnotobiotic culture conditions can be used as a powerful experimental approach to study the contribution of bacteria to the *Artemia* diet. The results of *Artemia* performance in terms of survival and body length illustrate that bacteria can be a direct food source for *Artemia*. Moreover, joint feeding of bacteria and other food sources (e.g. microalgae and baker´s yeast) may result in a beneficial effect on nutrient assimilation. This was demonstrated using diets combining different food sources on a dry weight basis, though other experimental approaches (such as isonitrogenous diets) could be used.

Bacterial mass, stimulated by the addition of preferentially a simple carbohydrate source at low apparent C/N ratios (C/N 10), can be a dietary substitute of microalgae for *Artemia* in prolonged conventional xenic culture conditions when algal supply is limited. Up to 67% of microalgae could thus be substituted by heterotrophic bacteria without negative effect on *Artemia* biomass production.

Manipulation of C/N ratio in *Artemia* culture systems may help to convert the organic waste from *Artemia* into bacterial biomass, which in turn can be used as the food source for *Artemia*. Promoting bacterial growth may also contribute in water quality

control. It leads to increased sustainability and environmentally friendly aquaculture systems.

The combination of promoting bacterial growth and *Artemia* culture can be an effective model in extractive aquaculture effluent management.

Our results, however, still remain to be confirmed in pond culture conditions. The size of flocs should be measured using different carbohydrate sources and C/N ratios, to find out conditions resulting in optimum floc size for *Artemia*. Moreover, in pond conditions ambient temperature and salinity may affect the microbial community composition and the bacterial production rate (Maicá et al., 2011). Bacterial growth is often slow in highly saline water (Kaye and Baross, 2004), and the salinity of water in *Artemia* culture ponds is normally maintained at around 100 g/L. Bacterial diversity is generally inversely related to salinity, with for example only few *Salinivibrio* sp. species dominating at 100 g/L salinity (Tkavc et al., 2011). Stimulation of bacterial growth may thus be more successful in the fertilizer ponds where water salinity is around 35 g/L, and from where bacteria-rich water may be pumped to the *Artemia* ponds.

Stimulation of bacterial growth may also have an effect on dissolved oxygen levels and water pH, as aeration is not applied in *Artemia* culture ponds and as dissolved oxygen is also needed for the respiration of heterotrophic bacteria. Low dissolved oxygen levels and pH may have a negative effect on survival and growth of *Artemia*. Therefore, promoting bacterial growth could be more efficiently performed in small ponds, where an aeration system can easily be installed to ensure saturated levels of dissolved oxygen for optimal *Artemia* growth.

If promoting bacterial growth in large pond-based *Artemia* culture systems, carbohydrate addition should be applied at windy days and by day time to ensure sufficiently high dissolved oxygen levels. Moreover, slowly degrading carbohydrate sources such as rice bran, soy meal etc., are recommended to avoid oxygen depletion by the massive growth of bacteria. Extra raking activities should be applied at night when oxygen depletion may occur in the culture ponds; disturbance of the water column by raking increases air-to-water contact and transfer of oxygen from the air into the water. Additionally, raking resuspends food particles from the bottom

into the water column and disturbs growth of benthic algae, and may thus have a major influence on microbial floc formation.

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Reference list

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Summary

Summary

Trying to meet the demand of *Artemia* products for aquaculture, the technique of *Artemia* culture in salt ponds has been introduced throughout the world. In the typical management, phytoplankton, which is stimulated to grow by inorganic and organic fertilizers, is used as a main food source and rice bran and soybean meal are mostly used as supplemented feed for *Artemia*. However, the contribution of naturally occurring bacteria in the culture systems to the *Artemia* diet is generally overlooked. In open *Artemia* culture systems uncontrollable variations in many parameters may occur, making it extremely difficult to evaluate the contribution of a single diet (bacteria) among a wide variety of foods (algae, bacteria, detritus particles…) available at the same time. The gnotobiotic culture system, meaning that only known micro-organisms are present, can be an excellent tool to investigate the effect of microbiota on the organism being studied. Additionally, ^{15}N stable isotope or fatty acid analysis can be used as tools to follow the fate of bacteria in *Artemia*.

Nine isolates (coded from H1 to HT9), originating from a well performing *Artemia* culture, were daily offered to axenically hatched second instar of *Artemia franciscana* at $5x10^6$ cell/mL over a 6 days experimental culture period under gnotobiotic culture conditions. LVS3 (*Aeromonas hydrophila*) with proven positive effects on *Artemia* was also used alongside as positive control to compare the effects of the above isolates on *Artemia* larvae. The results revealed that among nine isolates *Artemia* could survive on HT3, HT6, HT7 and HT8 (chapter 2). Particularly, the survival and body length of HT3 and HT6 fed *Artemia* was higher or identical to that obtained in reference LVS3 fed *Artemia*. Through sequencing HT3 and HT6 were identified with 99% similarity with *Tamlana* sp. ZJU HZ22 and *Bacillus subtilis* 168, respectively.

To sustain good animal growth, nitrogen is quantitatively the most important nutrient. It is a major constituent of essential compounds such as proteins. In a subsequent step, the effect of heterotrophic bacteria and microalgae or yeasts on the growth of *Artemia* was evaluated by studying N assimilation. The N assimilation from the food in *Artemia* was measured by using the ^{15}N stable isotope tracer as the study tool. HT3 and HT6 as described above were selected, based on their higher positive effect on *Artemia* performance as compared to the other isolates. HT3 and HT6 were

co-fed with one of two microalgae strains, *Dunaliella tertiolecta* DT CCAP 19/6B (DT 19/6B) and DT CCAP 19/27 (DT 19/27). Analogously HT3 and HT6 were co-fed with one of two strains of baker´s yeast *Saccharomyces cerevisiae*, either WT or its mutant mnn9. Each combination of algae or yeast and bacteria was offered for 24 h to second instar *Artemia* in different proportions, i.e. 10/90, 50/50 and 90/10% on a dry weight basis, with the total amount of food supplied being based on a reference *Dunaliella tertiolecta* mono-diet. Mono-diets consisting of 100% algae, 100% yeast and 100% bacteria were added as controls. One of the food sources (e.g. bacteria, yeast or microalgae) in the mixed diets was labeled with ^{15}N. Precautions were taken to prevent the ^{15}N labeled algal and bacterial cells from growing and multiplying: experimental conditions excluding photosynthesis were applied for microalgae and rifamycin was applied at 0.005 g/L for bacteria. In the experimental set-up with yeast, no specific precautions were taken because yeast *S. cerevisiae* doesn't grow in conditions deprived of organic carbon. The ^{15}N accumulation in *Artemia* tissue was measured after 24 h incubation in the food mixes.

The results revealed that the N assimilation from both strains of microalgae slightly increased or was identical as compared to the *Artemia* fed solely microalgae, although the concentration of microalgae was reduced with 10% and 50% in the mixed diets (chapter 2). But in the combinations of yeast and bacteria, the N assimilation from yeast was reduced when the concentration of yeast was reduced in the mixes (chapter 3). Moreover, the N assimilation from both yeast and microalgae strains in *Artemia* seems related to the bacteria strains used in the mixes. In the presence of HT3, the N assimilation from yeast and microalgae was less than in the presence of HT6.

Moreover, co-feeding with DT 19/6B always induced better N assimilation from bacteria than with DT 19/27. The presence of DT 19/6B increased N assimilation from both strains of bacteria, although the proportion of bacteria was reduced with up to 50%. The N assimilation from HT6 bacteria even nearly doubled when 10% of HT6 was replaced by DT 19/6B in comparison to solely HT6 fed *Artemia*. In the case of co-feeding with yeast, the presence of WT induced better N assimilation from HT3 than mnn9.

Finally, we examined the suitability of bacterial mass as a substitute for microalgae in prolonged conventional xenic *Artemia* cultures. In this study carbohydrate addition

in the *Artemia* culture, using different C/N ratios and carbohydrates sources aimed to stimulate the conversion of nitrogen in the culture medium into heterotrophic bacteria biomass. The ^{15}N and fatty acids signature were used as study tools to follow the fate of N and fatty acids assimilation from bacteria in *Artemia*, respectively. The assimilation of bacteria was determined by the addition of ^{15}N-nitrogen into the cultures to label bacteria. Conditions excluding photosynthesis, as used in the previous tests, could not be applied in the present study because many manipulations, such as feeding and waste removing, needed to be performed daily with illumination over a 15-day culture period. Therefore, intact but non-viable cells of *Tetraselmis* sp. were chosen as a reference food for *Artemia*, assuming that ^{15}N assimilation was not done by non-viable cells in light conditions.

In the first experiment (chapter 4), aiming to document 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, sucrose and soluble potato starch were used as carbon sources to obtain carbon/nitrogen (C/N) ratios 10 and 50 in *Artemia* cultures. The contribution of heterotrophic bacteria was assessed at two different algal feeding regimes: a standard feeding regime (SF), corresponding with *ad libitum* feeding, and a low feeding (LF) regime, corresponding with a quarter of the SF regime, were offered to *Artemia.* At the SF regime, the body length of *Artemia* slightly increased in the culture with C/N ratio 10 for both carbon sources, while it was reduced at C/N ratio 50 as compared to the culture without carbohydrate addition. A slightly higher *Artemia* performance was obtained with sucrose than with potato starch. However, massive growth of bacteria was observed when C/N ratio 50 was applied in the standard feeding ration, leading to poor performance of *Artemia*. The results further showed that carbohydrate addition significantly increased growth and total biomass production of *Artemia* in the LF regime for both carbohydrate sources, as compared to the same feeding ration without carbohydrate addition. Moreover, ^{15}N accumulation and levels of the fatty acids 16:1n-7 and 18:1n-7 in *Artemia* were higher in the cultures where bioflocs were stimulated to grow at low algal feeding regime as compared to those obtained in *Artemia* receiving the standard feeding regime.

In the second experiment (chapter 5), heterotrophic bacteria were stimulated to grow in *Artemia* cultures with progressively lower microalgae feeding rations, i.e. the

standard feeding ration SF1, half of SF1 (SF1/2), a third of SF1 (SF1/3) and a quarter of SF1 (SF1/4). The same algal strain *Tetraselmis* sp. as described above was offered to *Artemia*. Sucrose was used as carbon source to obtain a C/N ratio 10. Similarly to the previous study, the ^{15}N isotope and fatty acid analyses were used as tools to follow the fate of N and fatty acid assimilation from bacteria in *Artemia*, respectively. The results showed that the addition of sucrose at C/N ratio 10 increased the survival and total biomass production of *Artemia* in all feeding regimes, as in our previous study. Particularly, stimulation of bacterial growth in the SF1/3 treatment induced an improvement of the *Artemia* biomass production equal to that obtained in the culture where *Artemia* were offered solely algae at SF1 ration. Moreover, the levels of the fatty acids 16:1n-7 and 18:1n-7 and especially ^{15}N increased in the *Artemia* cultures where bacterial growth was stimulated by carbohydrate addition. The increase of fatty acid levels and ^{15}N was higher in *Artemia* receiving the low feeding regimes than in *Artemia* receiving the standard feeding regime, as in our previous study. This confirmed the results of the previous study that *Artemia* utilized relatively more bacteria at the lowest algae supply.

In conclusion, the results in the present study indicated that associated bacteria in *Artemia* culture systems can be a direct food source for *Artemia* and can result in improved N assimilation from other food sources in *Artemia*. *Artemia* takes dietary advantage from bacteria in algae-limited conditions. Moreover, manipulating the C/N ratio can reduce the algal ration supplied, and result in increased *Artemia* biomass production. Our results still remain to be validated in pond-based *Artemia* culture conditions, where typical management procedures (e.g. daily raking; lack of mechanical pond aeration) and ambient parameters, such as temperature, salinity and oxygen, may have a major influence on microbial production and on the production rate, size and bacterial composition of bioflocs.

Samenvatting

Samenvatting

Om te trachten aan de vraag naar *Artemia*-producten in de aquacultuur te voldoen, is de techniek van *Artemia*-kweek in zoutvijvers op vele plaatsen in de wereld geïntroduceerd. Bij het typisch management wordt de groei van fytoplankton, als voornaamste voedselbron, gestimuleerd door gebruik van anorganische en organische bemesters; gemalen rijstekaf en sojameel worden frequent gebruikt als voedselsupplementen voor *Artemia*. In welke mate de van nature voorkomende bacteriën in de kweeksystemen bijdragen tot het *Artemia*-deet, wordt meestal over het hoofd gezien. In open *Artemia*-kweeksystemen treden soms oncontroleerbare schommelingen in talrijke parameters op, hetgeen de inschatting van de bijdrage van één enkele voedselbron (bacteriën) tussen een groot aanbod aan voedsel (algen, bacteriën, detrituspartikels...) uiterst bemoeilijkt. Het gnotobiotische kweeksysteem (waarin alleen bekende micro-organismen aanwezig zijn) kan een uitstekend werkmiddel zijn om het effect van microbiota op het organisme in kwestie te onderzoeken. Bovendien kan de analyse van de ^{15}N stabiele isotoop of van vetzuren gebruikt worden als middel om na te gaan hoe de bacteriën in *Artemia* geassimileerd worden.

Negen isolaten (HT1-HT9), afkomstig uit een goed presterende *Artemia*-cultuur, werden dagelijks aangeboden aan axenisch ontloken instar II van *Artemia* franciscana aan $5x10^6$ cellen/mL gedurende een experimentele periode van 6 dagen bij gnotobiotische kweekomstandigheden. Daarnaast werd LVS3 (*Aeromonas hydrophila*), met een bewezen positief effect op *Artemia*, gebruikt als positieve controle om de effecten van hoger genoemde isolaten op de *Artemia*-larven te onderzoeken. De resultaten toonden aan dat van de negen isolaten *Artemia* kon overleven op HT3, HT6, HT7 en HT8 (Hoofdstuk 2). In het bijzonder bij voedering met HT3 en HT6 waren de overleving en lichaamslengte van *Artemia* hoger dan, of identiek aan, de waarden bekomen bij *Artemia* gevoed met de referentie LVS3. Door sequencing konden HT3 en HT6 met 99% gelijkenis geïdentifceerd worden als zijnde respectievelijk *Tamlana* sp. ZJU HZ22 en *Bacillus subtilis* 168.

Stikstof is kwantitatief het belangrijkste nutriënt voor een goede groei bij dieren. Het is een belangrijke bouwsteen van essentiële componenten zoals eiwitten. In een volgende stap werd het effect van heterotrofe bacteriën en microalgen of gistcellen bestudeerd op de groei van *Artemia*, door het volgen van de stikstofassimilatie. De stikstofassimilatie van het voedsel in *Artemia* werd gemeten, gebruik makend van de ^{15}N stabiele-isotooptracer. HT3 en HT6, hierboven beschreven, werden geselecteerd op basis van hun hoger positief effect op *Artemia*-performantie in vergelijking met de andere isolaten. HT3 en HT6 werden als voedsel aangeboden samen met één van beide stammen van de microalga *Dunaliella tertiolecta*, DT CCAP 19/6B (DT 19/6B) of DT CCAP 19/27 (DT 19/27). Op een analoge manier werden HT3 en HT6 aangeboden als voedsel met één van beide stammen van bakkersgist *Saccharomyces cerevisiae*, hetzij WT of zijn mutant mnn9. Elke combinatie van algen of gist en bacteriën werd als voedsel aangeboden gedurende 24 u aan instar II *Artemia* in verschillende verhoudingen, nl. 10/90, 50/50 en 90/10% op drooggewichtbasis. De totale hoeveelheid toegediend voedsel was gebaseerd op een referentie-monodieet van *D. tertiolecta*. Monodiëten bestaande uit 100% algen, 100% gist en 100% bacteriën werden ook opgenomen als controles. Eén van de voedsels (hetzij bacteriën, gist of microalgen) in de gemengde diëten werd gemerkt met ^{15}N. Voorzorgsmaatregelen werden genomen om te voorkomen dat algen- en bacteriëncellen, gemerkt met ^{15}N, zouden groeien en zich voortplanten: voor microalgen werden de experimentele condities zo gehouden dat fotosynthese uitgesloten was, en voor bacteriën werd rifamycine toegevoegd aan 0.005 g/L. In de experimentele set-up met gist werden geen speciale voorzorgsmaatregelen genomen omdat gist *S. cerevisiae* niet groeit in afwezigheid van organische koolstof. De ^{15}N accumulatie in het *Artemia*-weefsel werd gemeten na 24 u incubatie in de voedselmengsels.

De resultaten toonden aan dat de stikstofassimilatie van beide stammen microalgen enigszins toenam, of identiek was, in vergelijking met *Artemia*, alleen gevoed met microalgen, wanneer de concentratie aan microalgen verminderd werd met 10% en 50% in de gemengde diëten (Hoofdstuk 2). Maar in de combinaties gist-bacteriën was de stikstofassimilatie van gist lager wanneer de concentratie aan gist afnam in de mengels (Hoofdstuk 3). Bovendien schijnt de stikstofassimilatie van zowel gist als van microalgen in *Artemia* verband te houden met de bacteriële stammen die

gebruikt werden in de mengsels: in de aanwezigheid van HT3 was de stikstofassimilatie uit gist en microalgen lager dan in de aanwezigheid van HT6.

Bovendien leidde co-voeding met DT 19/6B altijd tot betere stikstofassimilatie uit bacteriën dan met DT 19/27. De aanwezigheid van DT 19/6B verhoogde de stikstofassimilatie uit beide bacteriële stammen, hoewel het aandeel bacteriën tot 50 % verminderd werd. De stikstofassimilatie uit HT6 bacteriën verdubbelde zelfs bijna wanneer 10% van HT6 vervangen werd door DT 19/6B, in vergelijking met *Artemia* uitsluitend gevoed met HT6. Bij co-feeding met gist leidde de aanwezigheid van WT tot betere stikstofassimilatie uit HT3 dan de aanwezigheid van mnn9.

Tenslotte onderzochten we de geschiktheid van bacteriële biomassa als een substituut voor microalgen in meer langdurige conventionele xenische kweek van *Artemia*. In deze studie werden carbohydraten toegevoegd aan de *Artemia*-kweek, gebruik makend van verschillende C/N- verhoudingen en carbohydraatbronnen, om de omzetting van stikstof in het kweekmedium tot heterotrofe bacteriële biomassa te bevorderen. De gehaltes aan ^{15}N en vetzuren werden gebruikt als middel om respectievelijk het lot van stikstof en de vetzuurassimilatie van de bacteriën naar *Artemia* te volgen. De assimilatie uit bacteriën werd bepaald door toevoeging van ^{15}N-stikstof in de kweek, om de bacteriën te merken. Kweekomstandigheden die fotosynthese uitsluiten, zoals gebruikt in de vorige tests, konden niet toegepast worden bij deze studie omdat talrijke manipulaties (zoals voederen, verwijderen van afvalstoffen) dagelijks bij belichting dienden verricht te worden gedurende een kweekperiode van 15 dagen. Daarom werden intacte maar niet-leefbare cellen van Tetraselmis sp. gekozen als referentievoedsel voor *Artemia*, in de veronderstelling dat ^{15}N assimilatie niet gebeurde door niet-leefbare cellen bij belichting.

Het eerste experiment (Hoofdstuk 4) had tot doel om de mogelijke positieve en negatieve effecten van bacteriële groei in het kweekmedium te onderzoeken, alsook het effect van de opname en assimilatie van bacteriën op de performantie van *Artemia*; sucrose en oplosbaar aardappelzetmeel werden gebruikt als koolstofbronnen om koolstof/stikstof (C/N)-verhoudingen van 10 en 50 te bekomen in de *Artemia*-kweek. De bijdrage van de heterotrofe bacteriën werd geëvalueerd bij twee verschillende algenregimes: een standaard voederregime (SF) corresponderend met *ad libitum* voederen, en een laag voederregime (LF),

corresponderend met een kwart van SF, werden aan *Artemia* aangeboden. Bij het SF-regime nam de lichaamslengte van *Artemia* licht toe in de kweek bij C/N-ratio 10 voor beide koolstofbronnen, terwijl de lengte afnam bij C/N-ratio 50 in vergelijking met de kweek zonder koolstoftoevoeging. Met sucrose werd een licht hogere *Artemia*-performantie bekomen dan met aardappelzetmeel. Maar massale groei van bacteriën werd waargenomen wanneer C/N-ratio 50 werd gebruikt bij het standaardvoederregime, hetgeen leidde tot lage *Artemia*-performantie. De resultaten toonden verder aan dat koolstoftoediening significant de groei en totale biomassaproductie van *Artemia* in het LF-regime verhoogde voor beide koolstofbronnen, in vergelijking met het zelfde voederregime zonder koolstoftoediening. Bovendien waren ^{15}N-accumulatie en de gehaltes aan de vetzuren 16:1n-7 en 18:1n-7 in *Artemia* hoger in de culturen waar de groei van biovlokken werd gestimuleerd bij laag algenvoederregime, in vergelijking met de waarden verkregen in *Artemia* bij standaard-voederregime.

In het tweede experiment (Hoofdstuk 5) werd de groei van heterotrofe bacteriën gestimuleerd in *Artemia*-kweken bij progressief afnemende algenvoederregimes, nl. het standaardregime SF1, de helft van SF1 (SF1/2), een derde van SF1 (SF1/3) en een vierde van SF1 (SF1/4). Dezelfde algenstam van Tetraselmis sp., als hierboven beschreven, werd aangeboden aan *Artemia*. Sucrose werd gebruikt als koolstofbron om een C/N-ratio 10 te bekomen. Zoals in de vorige studie werden de ^{15}N-isotoop en vetzuuranalyse gebruikt als middelen om respectievelijk het lot van N en de vetzuurassimilatie van bacteriën in *Artemia* te volgen. De resultaten toonden aan dat de toevoeging van sucrose bij C/N-ratio 10 de overleving en totale biomassa-productie van *Artemia* bij alle voederregimes bevorderde, zoals in onze vorige studie. In het bijzonder in de SF 1/3-behandeling leidde een stimulering van de bacteriële groei tot een verbetering van de *Artemia*-biomassaproductie tot op een niveau gelijk aan dat van de kweek waar *Artemia* alleen algen volgens het SF1-regime kreeg. Bovendien namen de gehaltes van de vetzuren 16:1n-7 en 18:1n-7, en vooral van ^{15}N, toe in de *Artemia*-culturen waar bacteriële groei werd gestimuleerd door koolstoftoevoeging. De toename van de vetzuurgehaltes en van ^{15}N was hoger bij *Artemia* die de lage voederregimes kregen dan bij *Artemia* in het standaardregime, zoals in onze vorige studie. Dit bevestigde de resultaten van de

vorige studie dat *Artemia* relatief meer bacteriën opneemt wanneer het aanbod aan algen laag is.

Tot besluit toonden de resultaten in de huidige studie aan dat geassocieerde bacteriën in *Artemia*-kweeksystemen een directe voedselbron voor *Artemia* kunnen zijn, en dat ze kunnen resulteren in verhoogde stikstofassimilatie in *Artemia* uit andere voedselbronnen. *Artemia* maakt voor zijn dieet gebruik van bacteriën wanneer het aanbod aan bacteriën beperkt is. Bovendien kan manipulatie van de C/N-ratio de hoeveelheid toegediende algen verminderen en resulteren in verhoogde productie van *Artemia*-biomassa. Onze resulten moeten nog bevestigd worden in *Artemia*-kweekomstandigheden in vijvers, waar bepaalde procedures typisch voor het management (bv. dagelijks harken van de bodem; afwezigheid van mechanische beluchting van de vijvers) en omgevingsparameters zoals temperatuur, saliniteit en zuurstof, een belangrijke invloed kunnen hebben op de microbiële productie en op de productiesnelheid, afmetingen en bacteriële samenstelling van de biovlokken.

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Curriculum vitae

Curriculum vitae

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2004: Master in aquaculture, Lab of Aquaculture & *Artemia* Reference Center, Faculty of Bioscience Engineering, Gent University. Thesis title: Beneficial effect of selected bacterial strains on axenically cultured *Artemia*. Promotor: Prof. Dr. Peter Bossier, supervisor: Jean Dhont

2008: PhD candidate, Lab of Aquaculture & *Artemia* Reference Center, Faculty of Bioscience Engineering, Gent University.

Professional activities

1997-2001:

- Research on production of *Artemia* cyst/biomass in the Vinh Chau salt fields, Mekong delta, southern of Vietnam.

- Attend the extension and transfer culture techniques of *Artemia* for farmers in Soc Trang and Bac Lieu provinces, Mekong delta, southern of Vietnam.

2001: Three months training (September to November) on *Artemia* biomass culture at Lab of Aquaculture & *Artemia* Reference Center, Faculty of Bioscience Engineering, Gent University.

2002-2004: Study MSc program at Ghent University, Belgium.

2005: Coordinator and lecturer of the international training course on *Artemia* at Cantho University, Vietnam: *Artemia* pond production & its use in aquaculture.

2005-2007:

- Study on the effect of some selected microalgae on *Artemia* biomass quality at Vinh Chau salt fields, Mekong delta, southern of Vietnam.
- Attend the extension and transfer *Artemia* culture techniques for farmers in Soc Trang and Bac Lieu salt woks, Mekong delta, southern of Vietnam.

2007 (September to November): Transfer *Artemia* culture techniques for the Marine Products Export Development Authority (MPEDA) staff scientists at Tharuvaikulam, Tuticorin, Tamilnadu, India.

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2011-2012: Nur Ahyani. Manipulation of C/N ratio and carbon source to stimulate the growth of bacteria as food for *Artemia* in a laboratory culture system. Master of Sciences in Aquaculture at the Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium

2012-2013: Janeth Rukanda. The use of bacteria as food for *Artemia* in labolatory based culture system. Master of Sciences in Aquaculture at the Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium

Participation in international scientific congress

Oral presentation (presentation given by **author in bold)**

Huynh Thanh Toi, **Bossier**, P., Van Stappen, G., Sorgeloos, P. The effects of manipulating C/N ratios on *Artemia* performance in an *Artemia* biomass production system. International workshop *Artemia* pond production: helping to solve the challenges of aquaculture in the 21st century, Can Tho University, Can Tho (Vietnam), 18-21 March **2012**

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Poster

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