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Assessment of a biofilm-based culture system within zero water exchange on water quality and on survival and growth of the freshwater shrimp *Neocaridina heteropoda heteropoda*

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Abstract

The contribution of biofilm to water quality and as a food source for the culture of the freshwater shrimp *Neocaridina heteropoda heteropoda* was assessed in indoor aquaria using a zero water exchange system. Two successive phases were conducted to evaluate biofilm development on different substrates (polyethylene net: PN, plastic bottles: PET, agrovelo: AV) and the effect of biofilm to shrimps culture. The biofilm grown on all substrates helped to keep a good water quality by the uptake of nitrogen compounds and the production of high levels of dissolved oxygen associated to the proliferation of autotrophic microorganisms. High survival, reproduction and hatching of shrimps were achieved in all groups mainly associated with good water quality. Final biomass of the shrimps was significant higher for PN and AV groups; while specific growth rate and the levels of lipids of the shrimps yielded similar values for all treatments indicating that the three substrates allowed the growth of a biofilm that resulted in a healthy food source with similar nutritional value for shrimps. The results show that the production of *N. heteropoda heteropoda* could be successfully conducted by a biofilm-based culture system with no water exchange, and thus contributing to a better water use. All materials tested were suitable substrates for biofilm growth, though AV and PET could reduce significantly production costs when compared to the PN. Moreover, by the recycling and reuse of waste materials (such as plastic bottles) could contribute to the development of a responsible, sustainable and environmentally friendly culture method.

Keywords: biofilm, artificial substrates, recyclable materials, water quality, *Neocaridina heteropoda heteropoda*, freshwater shrimp

Introduction

Crustacean production remains being one of the most important economic sectors of the global aquaculture (FAO 2012). The continuous growth of shrimp farming and the use of ornamental shrimps worldwide have led to an increase in the demand of these organisms justifying the need for further research of their biology and culture conditions. Culturing of several species of freshwater ornamental shrimps of the genders *Caridina*, *Neocaridina*, *Atya*, *Atyopis*, *Atyoida*, *Cherax* and *Macrobrachium* is carried out for commercial and educational purposes, and for restocking programs for native species (Werner 2003; Lin, Chang, Chen, Chiu, Wu & Chen 2006; De Grave, Cai & Anker 2008; Turkmen & Karadal 2012).

Recent political and social demands for responsible, sustainable and ecological friendly aquaculture has required the search of alternative farming systems that ensure low risk of contamination and minimal environmental impact. In recent years, marine and freshwater shrimp aquaculture practices have focused on the development of a periphyton-based culture system because of its relevance in natural aquatic ecosystems. In this context, periphyton (also referred as biofilm in the literature) is defined as a complex community of microorganisms attached to submerged substrates surfaces (Wetzel 1983; O'Toole, Kaplan & Kolter 2000). Biofilm has been shown to be greatly important in natural environments for various reasons: contributes to carbon fixation and nutrient cycling, it is an indicator of environmental changes, it is used to improve the water quality in lakes and reservoirs, and it can provide food for fishes, crustaceans, mollusks and others organisms (Azim, Beveridge, van Dam & Verdegem 2005). Coat, Lefançois, Lepoint, Vachiéry, Gros and Monti (2011) showed the importance of biofilm as food source in the diet of tropical shrimps (Palaemonidae, Atyidae and Xiphocarididae) inhabiting Caribbean rivers; for which biofilm percentage in the diet reached 85% for atyid shrimps, 29% for xiphocaridid shrimps and 14% for palaemonid shrimps.

In marine shrimps production, the biofilm-based culture systems with low or no water exchange showed improve water quality by recycling and recovering nutrients, and thus improving yield and reducing the use of water, and waste water discharge to the environment (Ballester, Wasielesky, Cavalli & Abreu 2007; Holl, Otoshi & Unabia 2011; Audelo-Naranjo, Martínez-Córdova, Gómez Jiménez & Voltolina 2012; Viau, Moreira Souza, Wasielesky, Abreu & Ballester 2013; among others). For freshwater crustacean culture, the effect of biofilm as an alternative and/or a complement food source in the culture has only been studied in the giant prawn *Macrobrachium rosenbergii* (Asaduzzaman, Wahab, Verdegem, Huque, Salam & Azim 2008; Uddin, Azim, Wahaba & Verdegem 2009; Hasan, Rahman, Hosen & Bashar 2012) and in the crayfish species *Cherax destructor* (Jones & Thanuthong 2002) and *Cherax quadricarinatus* (Viau, Ostera, Tolivia, Ballester, Abreu & Rodríguez 2012). However, no studies have been previously reported on the potential benefits of biofilm on the culture of freshwater shrimps.

Moreover, a variety of biodegradable and non-biodegradable materials as substrates for biofilm development have been tested to enhance shrimp, prawn and crayfish production. The substrates tested were artificial materials such as: polyethylene net (Ballester *et al.* 2007; Fernandes Da Silva, Ballester, Montserrat, Geracitano, Wasielesky & Abreu 2008; Preto, Pissetti & Wasielesky Jr.W., Poersch L.H. & Cavalli. R.O. 2009; Viau *et al.* 2012, 2013), PVC pipes (Thompson, Abreu & Wasielesky 2002; Khatoon, Yusoff, Banerjee, Shariff & Sidik Bujang 2007), plastic sheets (Khatoon *et al.* 2007) and the custom designed material AquaMats™ (Otoshi, Montgomery, Matsuda & Moss 2006; Audelo-Naranjo *et al.* 2012; Huang, Wan, Song & Hallerman 2013; among others). Among natural material, the bamboo was the most commonly tested as substrate for biofilm development (Uddin *et al.* 2009).

Neocaridina heteropoda heteropoda (Liang 2002) var. red or 'red cherry' is a freshwater Asiatic *Atyidae* shrimp with great commercial potential as an ornamental species. This species lives in small streams or lakes rocky bottoms, with dense aquatic vegetation and dead wood. Their digestive system has been described by Barbier (2010) and Bauer (2004) as being morphologically adapted for the consumption of both detritus and plankton. A field study revealed that *N. heteropoda heteropoda* is an omnivorous species and its diet includes mainly phytoplankton and detritus (Cabrita 2012). However, the use of biofilm on the red cherry shrimp culture under experimental conditions has not been previously reported.

Therefore, this study was conducted to evaluate the contribution of a biofilm-based culture system within zero water exchange on the survival, growth and reproduction of *N. heteropoda heteropoda*, as well on the water quality. Three materials were tested as artificial substrates for biofilm development considering the availability, costs and environmental impact with the aim of developing a sustainable and ecological friendly culture technique.

Materials and methods

Experimental design

The study was carried out in two successive experimental phases: the first phase comprised biofilm development on the artificial substrates while the

second phase evaluated the contribution of biofilm to the culture of the red cherry shrimp *N. heteropoda heteropoda*. Each experimental phase lasted 63 days and was conducted at the Central Bioterio of the FCEyN, University of Buenos Aires, Buenos Aires, Argentina (34° 36 W, 58° 23 S).

First experimental phase: biofilm development on different artificial substrates

Twelve 60 L glass aquaria (0.24 m^2 bottom surface) filled with continuously aerated dechlorinated tap water were kept during 63 days with zero water exchange. Only water lost by evaporation (8% of the total volume) was added weekly to each aquarium. The aquaria were housed in the Bioterio without environmental controls therefore they were exposed to natural ambient temperature and photoperiod (~14:10 L: D) throughout the experimental period.

Three artificial materials (four replicates of each one) were tested as substrates for the development of the biofilm: (i) polyethylene net (PN), (ii) agrovelo (AV) and (iii) plastic bottles (PET). PN is a high density polyethylene net (1 mm mesh size) frequently used in biofilm-based culture systems for crustacean production during the last decade, replacing the commercial designed substrate Aqua-Mats® (Meridian Applied Technology Systems, Calverton, MD, USA). AV is a polypropylene nonwoven substrate manufactured with superimposed layers of filaments randomly oriented (weight of 17 g/m^2). It is widely used in agriculture to protect crops from cold and frost; the relatively low-cost and high availability makes it a good material for aquaculture application. PET or polyethylene terephthalate is a commonly used material for carbonated beverage and water bottles.

Each aquarium assigned to PN, AV and PET groups had four pieces (25 × 17 cm, wide and high respectively) of each artificial material as substrate to allow the development of the biofilm. The pieces were placed vertically in the water column and were attached to a string in the upper and in the bottom of each aquarium. At the beginning of the trial, an inoculum of water rich in microorganisms (15% of the aquaria water volume) taken from a natural water body was added to each aquarium to promote the formation of the biofilm. In addition, 20 mg/aquarium/day of a commercial food for tropical fish (TetraColor® containing 47.5% crude protein, 6.5% lipid, 2.0% fibre, 6.0% moisture and 1.5% phosphorus) were added during the first 2 weeks of the experiment as a source of nitrogen and phosphorous to promote the development of the biofilm (Viau *et al.* 2012).

Second experimental phase: contribution of biofilm to the culture of *N. heteropoda heteropoda*

Once the biofilm was developed on the pieces of the artificial substrates, they were transferred to twelve plastic aquaria (0.1 m^2 bottom surface) assigned to PN, AV and PET groups in the presence of *N. heteropoda heteropoda*. For this purpose, indoor aquaria in a zero water exchange system were used filled with 20 L of continuously aerated water (60% of dechlorinated tap water and 40% of water from the aquaria where the biofilm was previously developed). Only the water lost by evaporation (8% of the total volume) was added to each aquarium once a week. All aquaria were previously stocked with 13 adults of *N. heteropoda heteropoda* corresponding to a density of 174.7 shrimps/m^2, with an overall mean body mass of 16 ± 8 mg (N = 156). No commercial diet was offered to the shrimps, thus the biofilm from the artificial substrates was the only food source available for the animals throughout the experiment.

Variables analysed

Biofilm sampling and monitoring

Biofilm development was monitored throughout both experimental phases. For this purpose, once a week (in the first phase) and once every 2 weeks (in the second phase) three small samples (2 × 2 cm) of the artificial substrate from each aquarium were removed to determine the biofilm dry weight, chlorophyll *a* concentration and to characterize the microorganisms.

For determination of dry weight, the biofilm was removed from each sample with a soft brush and placed in a vial and dried in stove at 60°C until constant weight. The biofilm dry weight was determined by the difference in the weight before and after dried (precisio ± 0.1 mg). For evaluating chlorophyll *a* concentration, the biofilm was removed from the sample with a soft brush and placed in a glass vial containing 5 mL acetone in total darkness for 24 h. Chlorophyll *a* concentration was determined in a spectrophotomer (JASCO Model 7850 UV-VIS) at 665 nm, before and after the acidification with HCl 0.1 N, according to the

equations of Marker, Nusch, Rai and Riemann (1980).

To characterize microorganisms, the biofilm was detached from the sample with a soft brush and an aliquot was analysed under a binocular microscope (Zeiss Imager.A1 AX10, 100 to 1000 × magnifications) and the main organisms present were identified on previously published identification schemes (Krammer & Lange-Bertalot 1986, 2004; Canter-Lund & Lund 1995; Hartley 1996; Lee, Leedale & Bradbury 2000).

At the end of the second experimental phase, the biofilm was completely removed from the substrates for determination of final biomass and the content of carbohydrates (using the phenol-sulphuric acid method described by Dubois, Gilles, Hamilton, Rebers & Smith 1956), lipids (according to Folch, Lees & Stanley 1957 Fring & Dunn 1970) and proteins (according to Bradford 1976).

Water quality variables

The following variables were monitored weekly during both experimental phases: temperature (thermometer, precision ± 0.5°C), pH (TRACER LaMotte® pH Meter, precision ± 0.01), dissolved oxygen (Digital Oxygen Meter, precision ± 0.01 mg/L), light intensity (Digital Lux Meter HDT 11308, precision 5%), ammonium (Wiener kit, Wiener Laboratorios S.A.I.C., Rosario, Argentina), nitrites (Acuanalítica kit, Acuanalítica®, Buenos Aires, Argentina) and nitrates (according to Armstrong 1963; APHA 1993).

Growth and survival performance of the cultured shrimps

At the end of the second phase, both survival and body wet weight of all shrimps (precision ± 0.1 mg) were determined. Growth was calculated as follows:

$$\text{Specific growth rate (SGR), expressed as \% day}^{-1} = [(\ln \text{final body mass} - \ln \text{initial body mass})/(\text{final time} - \text{initial time})] \times 100);$$

$$\text{Biomass, expressed as g m}^{-2} = [\text{sum of total body mass of shrimps}/\text{surface of the aquarium}]$$

In addition, the total shrimp biomass was used for determination of total lipids (according to Folch *et al.* 1957; Fring & Dunn 1970) for both adults and juveniles.

Statistical analysis

Mean final values of the biofilm dry weight, chlorophyll *a* concentration, water quality parameters, as well as survival, growth and the content of carbohydrates, lipids and proteins were analysed by a one way-ANOVA. Lilliefors and Levene tests were used for checking normality and homogeneity of variance respectively. Angular transformation was used for survival. Kruskall–Wallis non-parametric test was used for the analysis of data when it did not meet the assumptions of the analysis of variance. When pertinent, a *post hoc* Tukey's test was used for multiple comparisons of means. A 5% significance level was always considered (Sokal & Rohlf 1995).

Results

First experimental phase: biofilm development on different artificial substrates

Biofilm sampling and monitoring

For all experimental groups, an increase in chlorophyll *a* concentration of the biofilm was observed during the first month of the experiment; after that the values remained approximately constant during the rest of the study. No significant differences between the groups ($P > 0.05$) were found after 63 days (Fig. 1).

The biofilm composition was comprised for the following main groups of microorganisms: cyanobacteria, diatoms, chlorophytas, flagellates, ciliates, rotifers and nematodes (Table 1). During the first month, autotrophic organisms (such as, chlorophytas, diatoms and cyanobacteria) proliferated on the three tested substrates; while during the last month of the experiment heterotrophic organisms (mainly cilliates, nematodes and rotifers) were observed. All experimental groups revealed a similar settling pattern of microorganisms; nevertheless AV showed an apparent greater prevalence of those organisms with respect to the PN and PET groups.

Water quality variables

Mean values of abiotic parameters monitored during this phase shown a similar pattern among experimental groups. Temperature, pH, dissolved oxygen, light intensity and the nitrogen compounds did not show significant differences ($P > 0.05$) between treatments at the end of the

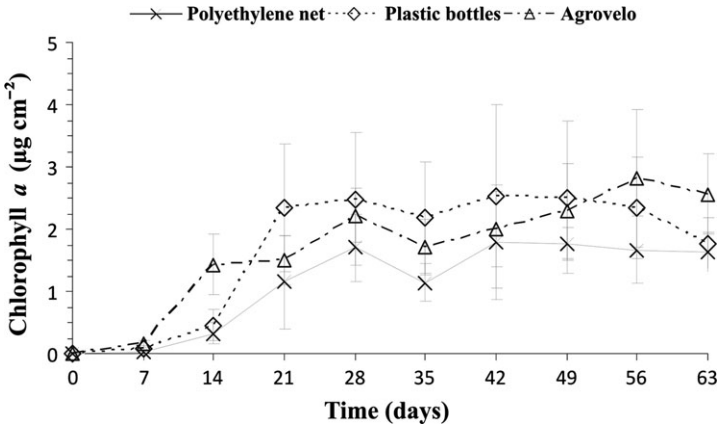

Figure 1 Values (mean ± SE) of chlorophyll *a* of the biofilm developed on different artificial substrates (polyethylene net, plastic bottles and agrovelo) during 63 days in a zero water exchange culture system.

63 day experiment (Table 2). During the experiment temperature ranged between 23 and 29°C; pH fluctuated between 7.8 and 9.6; dissolved oxygen between 7.8 and 9.4 mg L^{-1} and light intensity varied between 0.8 and 14.5 klx for all groups. During the first weeks of the experiment, an increase in the concentrations of ammonium, nitrites and nitrates was observed (Fig. 2). However, all treatments showed minimum values of the nitrogen compounds towards the end of the experimental phase.

Second experimental phase: contribution of biofilm to the culture of *N. heteropoda heteropoda*

Biofilm sampling and monitoring

Both chlorophyll *a* concentration and dry weight of the biofilm that adhered to the substrates showed a similar pattern throughout the experiment with no significant differences ($P > 0.05$) between the PN and PET groups at the end of the 63 day experiment. In the AV group, a great increase in both chlorophyll *a* concentration and dry weight of biofilm was detected during the last weeks of the experiment reaching significantly higher ($P < 0.05$) final values compared to the PN and PET groups (Fig. 3).

The main microorganisms present in the biofilm were the same as those observed during the first experimental phase with a great presence of heterotrophic organisms, such as cilliates, nematodes and rotifers (Table 1). The treatments showed a similar settling pattern; however, the AV group showed a higher occurrence of microorganisms throughout the experiment in comparison to the PN and PET groups.

At the end of the experiment the biomass and the content of total lipids, proteins and carbohydrates of the biofilm were significant higher ($P < 0.05$) for AV group as compared to PN and PET groups, without significant differences ($P > 0.05$) between these last two groups (Table 3).

Water quality variables

Mean values of water temperature, pH, dissolved oxygen and light intensity did not show significant differences ($P > 0.05$) between experimental groups after 63 days, revealing a similar pattern throughout the experiment. For all groups, temperature ranged between 21 and 30°C; pH varied between 8.2 and 9.3; dissolved oxygen ranged between 7.1 and 9.6 mg L^{-1} while light intensity fluctuated between 1.4 and 28.9 klx (Table 4).

Towards the end of the assay a significant increase ($P > 0.05$) in the ammonium and nitrite concentrations were observed in PET when compared to PN and AV groups (Table 4, Fig. 4). Whereas the nitrates concentration exhibited a similar pattern during the 2 months for all groups; although the final value of AV group was significantly ($P < 0.05$) lower than the values observed for the PET and PN groups (Table 4, Fig. 4).

Growth and survival of N. heteropoda heteropoda

Survival of the adult shrimps was not statistically different ($P > 0.05$) among treatments, stressing the high survival obtained for all experimental groups (Table 5). Ovigerous females were observed throughout the experiment in all treatments indicating that reproduction occurred successfully in all groups. The biomass of juveniles hatched was

Table 1 Main microorganisms characterized in the biofilm adhered to different artificial substrates (PN = Polyethylene net, PET = Plastic bottles, and AV = Agrovelo) in a zero water exchange system during four months of the experiment that was divided into two consecutive experimental culture phases (the first phase without shrimps, and the second phase with the presence of *Neocaridina heteropoda heteropoda* shrimps). (+): indicates presence of the microorganism at the time of sampling (0, 14, 28, 42 and 56 d) for each phase

			First experimental phase (biofilm without shrimps)															Second experimental phase (biofilm with presence of shrimps)													
			PN					PET					AV					PN					PET					AV				
Division	Family	Genus	0	14	28	42	56	0	14	28	42	56	0	14	28	42	56	0	14	28	42	56	0	14	28	42	56	0	14	28	42	56
Cyanophyta	Oscillatoriaceae	Not identified	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Chroococcales	Not identified	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bacillariophyta	Bacillariophyceae	Not identified	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chlorophyta	Chlorophyceae	*Oedogonium sp.*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Scenedesmus sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+		+	+	+	+	+
		Golenkinia sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+				+	+	+		
		Pediastrum sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				+	+				+	+	+	+	
		Ankistrodesmus sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+			+	+	+	+	+
	Zygnematophyceae	*Cosmarium sp.*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+			+	+	+	+	+
Rhizopoda (amoebas)		Not identified	+	+	+	+			+		+		+	+	+	+	+		+		+		+	+		+		+		+	+	
Mastigophora (flagelates)		Not identified	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ciliophora (cilliates)	Vorticellidae	*Vorticella sp.*				+	+		+	+	+	+		+	+	+	+	+	+	+	+		+	+	+			+	+	+	+	+
	Operculariidae	*Opercularia sp.*					+				+	+					+	+	+				+	+				+	+	+	+	
	Euplotidae	*Euplotes sp.*		+	+	+	+				+	+		+	+	+	+	+	+	+	+		+	+	+			+	+	+	+	+
	Holostichidae	*Holosticha sp.*				+	+			+	+	+		+		+	+	+	+	+			+	+	+			+			+	+
	Parameciidae	*Paramecium sp.*					+				+	+				+	+	+	+				+	+				+	+	+		
Actinopoda (heliozoans)	Raphidiophryidae	*Raphidiophrys sp.*	+	+	+	+		+	+				+	+	+																	
Rotifera	Not identified	Not identified			+	+	+			+	+	+		+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+
Nematoda	Not identified	Not identified				+	+					+			+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	+

Table 2 Values (mean ± SE) at day 63 of water quality parameters in the aquaria containing different artificial substrates (polyethylene net, plastic bottles and agrovelo) with the presence of biofilm in a zero water exchange culture system. Similar letters indicate absence of statistical significant differences ($P > 0.05$) among the treatments.<LD indicates below the limit of detection

	Experimental group		
Variable	**Polyethylene net**	**Plastic bottles**	**Agrovelo**
Temperature (°C)	27.8 ± 0.3[a]	27.7 ± 0.3[a]	27.8 ± 0.3[a]
pH	8.2 ± 0.1[a]	8.3 ± 0.1[a]	8.3 ± 0.1[a]
Dissolved oxygen (mg L^{-1})	8.4 ± 0.1[a]	8.5 ± 0.1[a]	8.5 ± 0.1[a]
Light intensity (klx)	2.2 ± 1.1[a]	3.4 ± 1.4[a]	2.6 ± 0.7[a]
Ammonium (mg L^{-1} NH_4^+)	<LD	<LD	<LD
Nitrites (mg L^{-1} $NO2^-$)	<LD	<LD	<LD
Nitrates (mg L^{-1} NO_3^-)	0.8 ± 0.3[a]	0.5 ± 0.2[a]	0.8 ± 0.2[a]

significantly ($P < 0.05$) higher in the AV and PN groups. No significant differences ($P > 0.05$) were detected for final body weight and specific growth rate among the groups after 63 days; while the final biomass was significantly ($P < 0.05$) higher for the AV and PN groups, for both juveniles and adults shrimps (Table 5).

Total lipids of the cultured shrimps showed no significant differences ($P > 0.05$) between groups, for both juveniles and adults (Table 5). Nevertheless, juveniles shrimp from the PET group had insufficient biomass for such determination.

Discussion

The biofilm helped to keep a good water quality in all experimental groups by the uptake of nitrogen compounds (ammonium, nitrites and nitrates) and the production of high oxygen levels. In addition, the biofilm was the only food supply for the shrimps which grew, reproduced and attained a high survival rate mainly associated to a good water quality. This study is the first evidence to demonstrate the usefulness of biofilm in the culture of the freshwater shrimp *N. heteropoda heteropoda* under experimental conditions. Its contribution represents a clear advantage for the maintenance of water quality, but most importantly, is it can serve as a single food source for the cultured shrimps, thus suggesting that a significant reduction in the production costs could be achieved.

During the first experimental phase that involved the growth of the biofilm, nitrogen compounds removal took place efficiently in all treatments. The ammonium, nitrites and nitrates concentrations oscillated in accordance to the biological activity of the biofilm developed on the three tested substrates. From the first month of the experiment, an increase in chlorophyll *a* concentration were observed in all groups correlated with the presence of some autotrophic microorganisms (such as chlorophytas, diatoms and cyanobacteria). The proliferation of these autotrophic algae allowed the recycling of nutrients in the culture water throughout this phase. In fact, at the end of the 63 days of the experiment minimum values of the nitrogen compounds were found for all treatments indicating that nutrient recycling by the microorganisms from the biofilm was achieved. In addition, dissolved oxygen concentration and pH were stable during the assay, assisting the growth of the microorganisms from the biofilm, and thus to the maintenance of the water quality. These results are consistent with those observed in other studies in which autotrophic organisms and nitrifying bacteria from the biofilm grown up on a substrate improved water quality mainly by sequestering the excess of toxic nitrogen compounds, such as ammonia and nitrite, and helped to maintain the dissolved oxygen concentration and the pH of the water in ponds (Azim, Wahab, Verdegem, van Dam, van Rooij & Beveridge 2002; Milstein 2005; Crab, Avnimelech, Defoirdt, Bossier & Verstraete 2007; Holl *et al.* 2011; Rios da Silva, Wasielesky & Abreu 2013).

At the second phase of the experiment, with shrimp *N. heteropoda heteropoda* present in the aquaria as well as the biofilm, physical and chemical water parameters showed a similar pattern among treatments remaining within the acceptable

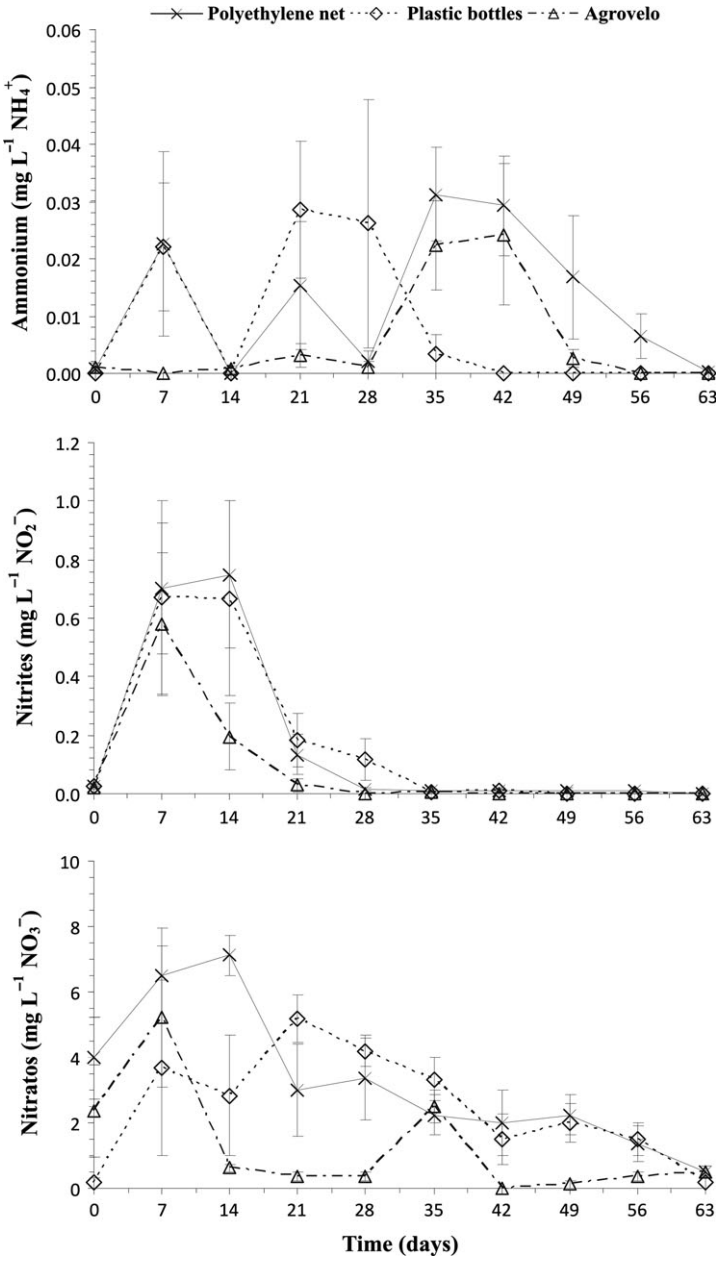

Figure 2 Values (mean ± SE) of ammonium, nitrites and nitrates in the aquaria containing different artificial substrates (polyethylene net, plastic bottles and agrovelo) in presence of biofilm during 63 days in a zero water exchange culture system.

limits established for this species culture (Barbier 2010). According to this author, *N. heteropoda heteropoda* var. red tolerates pH values between 6.8–9, dissolved oxygen concentrations as low as 5 mg L^{-1}, and temperatures between 23–27°C. During this study, mean temperature was 27°C, mean pH value was below 9 and dissolved oxygen concentrations remained above 8 mg L^{-1}. Light intensity showed fluctuations due to the variation in photoperiod.

An increase in the ammonium, nitrites and nitrates concentrations, were observed in all treatments as a result of the shrimps excretions. Even, during the last 2 weeks of experiment the ammonium and nitrite concentrations recorded for the PET group were significant higher in comparison to those in the PN and AV groups. However, the levels observed throughout the experiment for all treatments were within the values reported as safe for other crustaceans (Chen & Lei 1990; Chien

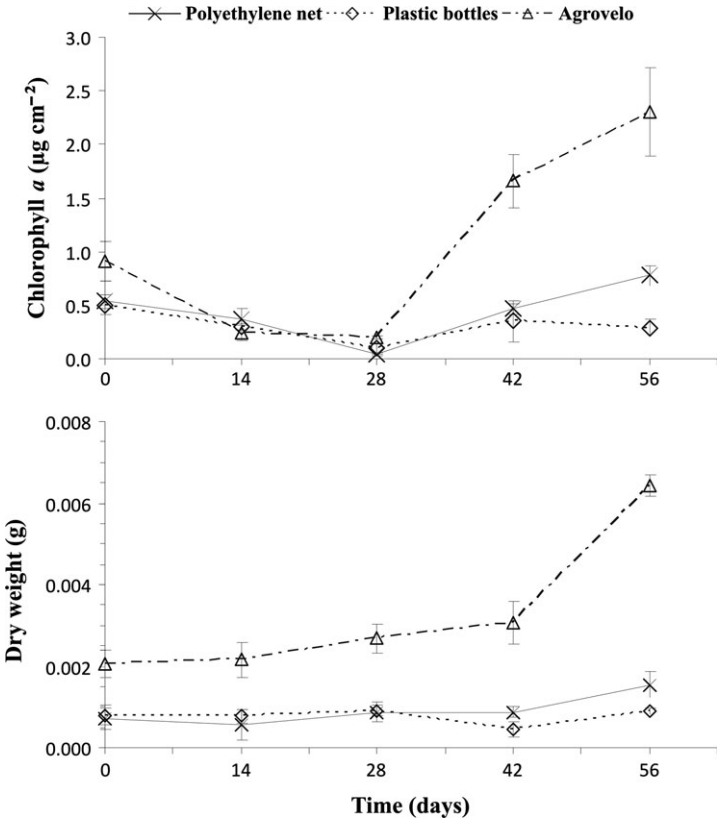

Figure 3 Values (mean ± SE) of chlorophyll *a* and dry weight of the biofilm adhered to different artificial substrates (polyethylene net, plastic bottles and agrovelo) during 63 days in a zero water exchange culture system with the presence of the shrimp *Neocaridina heteropoda heteropoda*.

Table 3 Final biomass and content (mean ± SE) of total lipids, protein and carbohydrates of the biofilm developed on different artificial substrates (polyethylene net, plastic bottles and agrovelo) after 63 days of the experiment. Different letters indicate statistical significant differences ($P < 0.05$) among the treatments

	Experimental group		
Variable	**Polyethylene net**	**Plastic bottles**	**Agrovelo**
Total biofilm biomass (g m^{-2})	2.9 ± 0.5^{a}	1.5 ± 0.6^{a}	15.6 ± 1.4^{b}
Lipids (mg g^{-1})	6.0 ± 0.4^{a}	6.9 ± 0.2^{a}	9.1 ± 0.6^{b}
Proteins (mg g^{-1})	3.4 ± 0.7^{a}	2.2 ± 0.7^{a}	6.9 ± 0.5^{b}
Carbohydrates (mg g^{-1})	11.3 ± 0.9^{a}	8.8 ± 0.7^{a}	14.1 ± 0.5^{b}

1992; Tomasso 1994; Ostrensky & Wasielesky 1995; Van-Wyk & Scarpa 1999; Holl *et al.* 2011). The toxicity of nitrogenous compounds for the species and even for the genus of these shrimps has not been established.

Chlorophyll *a* concentration of biofilm showed a decrease during the first month of the second experimental phase for all treatments probably due to the grazing of the biofilm by the shrimps. For PET group, the chlorophyll *a* levels remained low throughout this phase suggesting that the proliferation of the autotrophic community of microorganisms was not sufficient to regulate the uptake of the nitrogen compounds present in the water column. It is noteworthy that during the last month of the experiment a significant presence of cladocerans, ostracods and gastropods was found in the PET group. Although these organisms were also found in PN and AV groups, the gastropods (Gastropoda: Ancylidae) were observed attached only on the plastic bottle sheets and actively grazing on the biofilm in the PET treatment. This fact could explain the low levels of chlorophyll *a* concentration and low proliferation of microorganisms

Table 4 Values (mean ± SE) of water quality parameters in aquaria containing *Neocardina heteropoda heteropoda* shrimps cultured during 63 days in a zero water exchange system with the presence of biofilm adhered to three different artificial substrates (polyethylene net, plastic bottles and agrovelo). Different letters indicate statistical significant differences ($P < 0.05$) among the treatments. <LD indicates below the limit of detection

	Experimental group		
Variable	**Polyethylene net**	**Plastic bottles**	**Agrovelo**
Temperature (°C)	27.7 ± 0.1[a]	27.5 ± 0.2[a]	27.60 ± 0.18[a]
pH	8.9 ± 0.0[a]	8.6 ± 0.1[a]	9.1 ± 0.0[a]
Dissolved oxygen (mg L^{-1})	9.4 ± 0.2[a]	9.5 ± 0.1[a]	9.6 ± 0.1[a]
Light intensity (klx)	15.5 ± 1.2[a]	16.9 ± 2.5[a]	18.2 ± 0.8[a]
Ammonium (mg L^{-1} NH_4^+)	0.02 ± 0.004[a]	0.35 ± 0.18[b]	0.01 ± 0.003[a]
Nitrites (mg L^{-1} $NO2^-$)	< LD[a]	0.003 ± 0.002[b]	< LD[a]
Nitrates (mg L^{-1} NO_3^-)	1.3 ± 0.04[a]	1.8 ± 0.3[a]	1.0 ± 0.02[b]

recorded in the PET group. Several studies showed that periphytic biofilm may also be an important resource for invertebrate consumers, especially when phytoplankton is scarce (Van de Bund, Krips & Davids 1994; Siehoff, Hammers-Wirtz, Strauss & Ratte 2009; Cazzanelli, Forsstrom, Rautio, Michelsen & Christoffersen 2012). The probable explanation for the settlement of the gastropods on the plastic sheets of the PET group could be that the smooth surface of this material could serve as support for these organisms. Both the polyethylene net and agrovelo have textured surfaces that may difficult their settlement.

High survival was observed for all treatments and no mortality occurred in the AV and PN groups. Previous reports shown that survival of the marine shrimps *Litopenaeus vannamei* (Moss & Moss 2004) and *Penaeus monodon* (Stuart, Melony, Sellars, Crocos & Coman 2006), as well the freshwater crayfish *Cherax quadricarinatus* (Viau *et al.* 2012, 2013) was improved by a better water quality associated to the presence of biofilm to the culture ponds. The use of biofilm in the aquaria in the presence of *N. heteropoda heteropoda* resulted in low levels of the nitrogen compounds and high dissolved oxygen that had a positive impact on the shrimps' health and productivity. The final body weight and growth rate (SGR) of the shrimps showed similar values in all the treatments after 2 months. Moreover, ovigerous females and juveniles hatched were observed in all groups during the last month of trial demonstrating that reproduction and breeding of juveniles is feasible to occur in this culture system. The lower final biomass of the PET group, for both adults and juveniles, could be explained by the competition for biofilm as food resource with the cladocerans, ostracods and gastropods. The consumption of biofilm by these organisms could lead to a reduction in food intake by the reared shrimps, thus resulting in lower yields. Nevertheless, the growth of the shrimps in the PET treatment was not affected since there were not significant differences in the final body weight and SGR between treatments as above mentioned. During the experiment, both juveniles and adults *N. heteropoda heteropoda* were seen actively grazing on the biofilm in all the groups indicating a good acceptance for this food source by the farmed shrimps.

Moreover, the biofilm formed on the three substrates supplied a similar nutritional value for shrimps demonstrated by similar levels of total lipids as an estimate of energy reserves. This result correlates with the recorded values of the growth rates (SGR), since both levels of total lipids and SGR yielded similar values for all treatments suggesting that no serious nutritional deficit seemed to have occurred in the shrimps only fed with biofilm. Furthermore, the levels of total carbohydrates, lipids and proteins of the biofilm were significant higher for the AV group; nevertheless these energetic reserves in the PN and PET groups were sufficient to sustain an adequate growth and reproduction of the farmed shrimps.

Only few studies have characterized the nutritional quality of biofilm in terms of protein and lipid contents. According to some authors (Thompson *et al.* 2002; Fernandes Da Silva *et al.* 2008; Khatoon, Banerjee, Yusoff & Shariff 2009; Saikia 2011; Becerra-Dórame, Martínez-Porchas, Martínez-Córdova, Rivas-Vega, López Elias & Porchas-Cornejo 2012) microorganisms present in the biofilm constitute an important source of natural food providing essential nutrients like polyunsaturated fatty acid

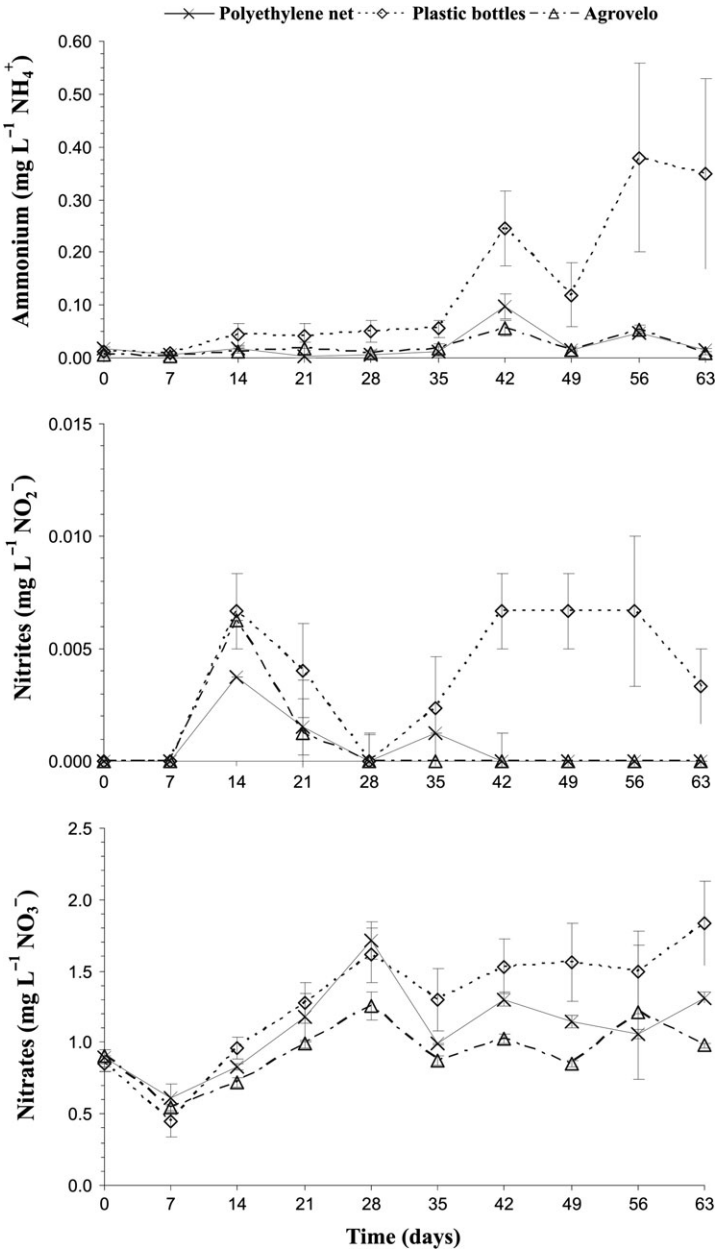

Figure 4 Values (mean ± SE) of ammonium, nitrites and nitrates in the aquaria containing shrimps of *Neocaridina heteropoda heteropoda* cultured during 63 days in a zero water exchange system with presence of biofilm adhered to different artificial substrates (polyethylene net, plastic bottles and agrovelo).

(PUFA), sterols, amino acids, vitamins and pigments that help to improve the development of raised animals. Fernandes Da Silva *et al.* (2008) characterized filamentous cyanobacteria, heterotrophic flagellates and nematodes presented in biofilm, as a relevant source of lipids, whereas diatoms could supply both protein and lipids of high nutritive value. Viau *et al.* (2012) observed a great accumulation of total lipids in the hepatopancreas of juveniles of *C. quadricarinatus* fed with biofilm, as well the presence of several microorganisms (e.g. filamentous cyanobacteria, diatoms, chlorophytes and xanthophytes) in their stomach content.

In the present experiment, it was no possible to estimate food consumption of both juveniles and adults through analysis of stomach content due to their small size and the high trituration of food items. However, microorganisms such as filamentous cyanobacteria, heterotrophic flagellates, diatoms, chlorophytes and nematodes were present in the biofilm developed in all three substrates throughout the experiment. Burford, Smith,

Variable	Experimental group		
	Polyethylene net	Plastic bottles	Agrovelo
Final body weight (mg)	56.0 ± 6.5[a]	47.9 ± 7.9[a]	52.0 ± 1.5[a]
SGR (% d^{-1})	2.1 ± 0.2[a]	1.8 ± 0.3[a]	2.0 ± 0.1[a]
Biomass (g m^{-2})			
Total	14.7 ± 1.0[a]	6.8 ± 0.5[b]	14.6 ± 1.1[a]
Adults	9.8 ± 1.1[a]	6.5 ± 0.4[b]	9.1 ± 0.3[a]
Juveniles	4.9 ± 0.5[a]	0.3 ± 0.1[b]	5.5 ± 0.9[a]
Survival (%)	100 ± 0.0[a]	82.1 ± 14.3[a]	100 ± 0.0[a]
Total lipids (mg g^{-1})			
Adults	5.4 ± 1.5[a]	5.0 ± 1.2[a]	5.1 ± 0.8[a]
Juveniles	28.7 ± 4.1[a]	–	32.2 ± 4.3[a]

Table 5 Values (mean ± SE) of final body weight, specific growth rate (SGR), biomass, survival and total lipids of *Neocaridina heteropoda heteropoda* shrimps cultured during 63 days in a zero water exchange system in the presence of biofilm adhered to three different artificial substrates (polyethylene net, plastic bottles and agrovelo). Different letters indicate statistically significant differences ($P < 0.05$) among the treatments

Tarbrett, Coman, Thompson, Barllay and Toscas (2004) using a stable isotope technique found that biofilm microorganisms can contribute 39–53% of total diet supplies of the postlarvae shrimp of *Penaeus monodon.* Likewise, Abreu, Ballester, Odebrecht, Wasielesky, Cavalli, Granéli and Anésio (2007) reported that biofilm can supply up to 80% and 70% respectively of the nitrogen demands of larvae and early juveniles of *Farfantepenaeus paulensis*. However, biofilm nutritional quality may vary according to the environmental conditions of the culture systems in which the substrate is assayed (Azim *et al.* 2002; Viau *et al.* 2013).

Interestingly, the agrovelo material showed the best results as substrate for biofilm development in terms of both food source and in the maintenance of water quality (essential for a good survival), although plastic bottles and polyethylene netting also were suitable substrates for biofilm growth in a zero water exchange system. Since agrovelo has a high specific surface area available for biofilm growth due to its complex texture in comparison to the non-textured surface such as the plastic bottles, the results may indicate that the specific surface area of the substrate is an important variable in determining biofilm growth. Nevertheless, it is important to consider several aspects in the choice of the materials used for aquaculture purposes. In this sense, a commercially available fibrous synthetic material AquaMats® (Meridian Aquatic Technology, LLC, Calverton, MD, USA) has been widely used for shrimps culture during the last decade because of its high-quality biofilm yield (Bratvold & Browdy 2001; Moss & Moss 2004; Audelo-Naranjo *et al.* 2012; Huang *et al.* 2013; among others); however, its high-cost is prohibitive for farmers. Instead, commercial polyethylene nets used as artificial substrate have replaced the AquaMats®, primarily because of their lower cost and widespread availability compared to the latter. These polyethylene nets as substrate for biofilm development have yielded good results in the culture of the marine shrimps *F. paulensis* (Thompson *et al.* 2002; Abreu *et al.* 2007; Ballester *et al.* 2007; Preto *et al.* 2009) and *F. brasiliensis* (Viau *et al.* 2013) and in the freshwater crayfish *C. quadricarinatus* (Viau *et al.* 2012).

The results of this study indicate that both agrovelo and plastic bottles could replace the polyethylene nets greatly reducing production investment due to the lower cost of these materials. Considering the current price of materials, the use of agrovelo leads to a reduction in approximately 60% in the production costs compared to the polyethylene net, while the use of plastic bottles has no cost since it is a recyclable waste material. In addition, substrate made from plastic bottles could be reused due to its high durability; while the characteristics of agrovelo make it unlikely that substrates made out of this material could be reused. Undoubtedly, it is important to promote responsible and environmentally friendly aquaculture practices. In this sense, the use of recyclable and reusable materials (e.g. plastic bottles) in a zero water exchange system may contribute to reduce environmental pollution and to a better use of water resources, thus resulting in a potential tool for the development of an economic, sustainable and ecological friendly culturing method. Regarding, we are currently working on improving the design of plastic material in order to achieve better results as substrate for biofilm growth.

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