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Nitrite-oxidizing bacteria, *Nitrospira*, distribution in the outer layer of the biofilm from filter materials of a recirculating water system for the goldfish *Carassius auratus*

Shiro Itoi, Noriaki Ebihara, Sayaka Washio, Haruo Sugita *

Department of Marine Science and Resources, Nihon University, Kameino, Fujisawa, Kanagawa 252-8510, Japan

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Abstract

We used a clone library method to investigate changes in the biofilm microflora associated with the conditioning of filter materials in a recirculating water system for the culture of goldfish *Carassius auratus*. The microbial density was higher in the outer layer of biofilm from filter materials (1.7×10^{10}–3.0×10^{10} cells/g) than in the inner layer (1.5×10^{9}–2.4×10^{9} cells/g) throughout the experimental period. The clone library method using bacterial 16S rRNA genes collected from the outer layer of filter material yielded sequences from four (day 8), nine (day 15), twelve (day 22) and nine (day 64) taxonomic categories of bacteria including Acidobacteria, Actinobacteria, Bacilli, Bacteroidetes, Flavobacteria, Fusobacteria, Nitrospira, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Sphingobacteria, Verrucomicrobia and unclassified bacteria. The inner layer yielded sequences from six (day 8), eight (day 15), five (day 22) and five (day 64) taxonomic categories of bacteria including Acidobacteria, Actinobacteria, Bacilli, Flavobacteria, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Sphingobacteria, Verrucomicrobia and unclassified bacteria. Bacteria in the outer layer of biofilm belonged predominantly to the genera *Acinetobacter*, *Cetobacterium*, *Comamonas*, *Flectobacillus*, *Flavobacterium*, *Ideonella* and *Pseudomonas*, whereas those in the inner layer were predominantly members of the genera *Flavobacterium*, *Flexibacter*, *Ideonella*, *Janthinobacterium*, *Pedobacter* and *Pseudomonas*. As the filter materials became conditioned, the population of nitrite-oxidizing bacteria related to *Nitrospira* was restricted to the outer layer of the biofilm. In addition, phylogenetic analysis indicated the presence of both an indigenous *Nitrospira* and a distinct type of *Nitrospira* that appeared after conditioning had begun.

Keywords: Nitrifying bacteria; *Nitrospira*; Recirculating water systems; Filter materials; Microbiota

1. Introduction

Nitrite-oxidizing bacteria are present in various environments including biotopes with moderate conditions (Bock and Koops, 1992), acid solids (Hankison and Schmidt, 1988; de Boer et al., 1991), soda lakes and alkaline soils (Sorokin et al., 1998), and permafrost and desert soils (Watson et al., 1986; Soina et al., 1991). In nitrite-oxidizing bacteria, four genera, *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira*, have been described to date. *Nitrobacter* and *Nitrococcus* are affiliated with the α- and γ-Proteobacteria, respectively (Teske et al., 1994), whereas *Nitrospira* belongs to the

Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

* Corresponding author. Tel./fax: +81 466 84 3679.
E-mail address: sugita@brs.nihon-u.ac.jp (H. Sugita).

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phylum Nitrospirae (Spieck and Bock, 2001). *Nitrospina* is listed in the δ-Proteobacteria (Teske et al., 1994; Radeva and Selenska-Pobell, 2005).

Nitrite-oxidizing bacteria convert nitrite to nitrate as part of the two-step process of nitrification, whereas ammonia-oxidizing bacteria are responsible for the conversion of ammonia to nitrite. The process of nitrification, including the role of nitrite-oxidizing bacteria can be studied in a recirculating water system. Recirculating water systems are widely used in fish rearing systems ranging from home aquaria to commercial aquaculture systems, providing optimal use of space, lower water requirements compared to stagnant water system, capacity for high stocking densities and a predictable and stable environment for the culture species. There have been only a few studies of the nitrifying bacteria in the microflora of circulating system filter materials (Kawai et al., 1964; Sugahara et al., 1974; Midlen and Redding, 1998). Studies have been hampered by low culturability of these environmental samples. Direct microscopic counts of bacteria in environmental samples, such as freshwater, seawater and sediments, often exceed viable counts by several orders of magnitude due to the presence of unculturable bacteria (Amann et al., 1995; Colwell and Grimes, 2000).

Recently, we reported the utility of clone library construction for assessing the bacterial composition of microflora, for example compositions of spirochetes and members of the genus *Vibrio* in the pufferfish *Takifugu niphobles* detected by clone library analysis were partly correlated with those counted by direct microscopic and viable counts, respectively (Shiina et al., 2006).

In a study of the microbial communities associated with well-conditioned filter material in recirculating water systems for carp *Cyprinus carpio* and goldfish *Carassius auratus* using the clone library method, we found that the bacterial communities on the surface of filter materials were composed of various bacterial groups and included a nitrite-oxidizer, *Nitrospira*, which comprised 8.0–9.8% of the bacterial taxa in the filter materials (Sugita et al., 2005). In this study, we investigated changes in microbial communities associated with the conditioning of filter materials in a recirculating water system used for goldfish culture and revealed differences in the microbial communities between outer and inner layers of biofilm from pebbles used as filter materials during the experimental period. In addition, phylogenetic analysis of the *Nitrospira*-related clones was carried out based on the sequence of 16S rRNA genes obtained at the clone library analysis.

2. Materials and methods

2.1. Experimental aquarium

A glass aquarium (60 × 35 × 30 cm) with a recirculating water system was filled with 50 L of distilled water. A total of 4.0 kg of pebbles (c.a. 5 mm diam.; 0.2 g/pebble) were subjected to heating at 400 °C for 3 h to remove most organic matter before being washed with distilled water. The pebbles were used as the filter material and the flow rate and temperature of the water were maintained at 6.6 L/min and 20 °C, respectively. Approximately 100 g of pebbles from another well-conditioned goldfish system was added to the filter bed of experimental aquarium. A goldfish weighing 11.8 g was maintained in the aquarium for 22 days during which nitrification proceeded well (Table 1). Ten goldfish (average body weight of 12.0 g) were then added to the aquarium and reared for an additional 1 month. All fish were fed commercial pellets (Super 8 for trout, Oriental Yeast Co., Ltd., Tokyo, Japan) at 1% body weight of fish per day. This system was considered well conditioned and no symptoms of disease were observed in fish during the experimental period. Concentrations of nitrate–nitrogen (NO_3–N) and nitrite–nitrogen (NO_2–N) were determined using the method of Strickland and Parsons (1972), whereas that of ammonium–nitrogen (total NH_4–N) was determined using phenol-hypochlorite colorimetric methods (Grasshof and Johannse, 1972) with unionized and ionized ammonia estimated manually using the method of Kawai et al. (1988).

2.2. Washing of filter materials

To estimate the recovery of microbial cells from filter materials, 5 g of pebbles were taken from the filter bed

Table 1
Water conditions for dissolved inorganic nitrogen, pH and dissolved oxygen in recirculating water systems of goldfish

Day†	Dissolved inorganic nitrogen (ppm)			pH	DO§ (mg/L)
	Ammonia‡	Nitrite	Nitrate		
3	0.217	0.000	0.238	7.8	7.35
13	0.053	0.730	0.875	8.0	7.35
20	0.082	0.010	1.595	7.9	8.25
59	0.250	0.050	2.564	6.1	6.13

†A goldfish (11.8 g) was maintained in the system for 22 days, and ten goldfish (average 12.0 g) were then added to the system and kept for an additional 1 month.
‡Concentrations of dissolved inorganic nitrogen as ammonia include those of ionized and unionized ammonia.
§DO represents concentrations of dissolved oxygen.