Monitoring of the bioencapsulation of a probiotic *Phaeobacter* strain in the rotifer *Brachionus plicatilis* using denaturing gradient gel electrophoresis

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Abstract

The bioencapsulation of the probiotic bacteria *Phaeobacter* 27-4 in the rotifer *Brachionus plicatilis* was monitored by culture methods and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA.

In a first experiment, the permanence of the probiotic bacteria in clear water and green water was studied. *Phaeobacter* 27-4 added to the water of the tanks (10^7 CFU ml^{-1}) remained at levels around 10^6 CFU ml^{-1} for 72 h and was not affected by the presence of the algae added (*Isochrysis galbana*, 10^5 cells ml^{-1}). The DGGE fingerprints showed a temporal predominance of the probiont in the water and the presence of bacteria belonging to the *Flavobacteria*, γ-*proteobacteria*, and *Sphingobacteria* groups. A *Tenacibaculum* strain became predominant when *Phaeobacter* 27-4 decline, and at the end of the experiment, bacterial profiles became similar to the initial ones with predominance of bacteria belonging to the *Oceanospirillaceae* family.

Three different ways of bioencapsulation of the probiont in the rotifer were assayed: *E24*, addition of *Phaeobacter* 27-4 for 24 h during the enrichment with *I. galbana*; *E3*, addition of *Phaeobacter* 27-4 during the last 3 h of the enrichment with *I. galbana* and *E3+*, with the bioencapsulation done in a separated step, after the 24 h enrichment with *I. galbana*, being the rotifers filtered, washed and transferred into tanks containing *Phaeobacter* 27- 4 in seawater, and maintained for 3 h.

The result showed that the presence of the algae was not determinant in the effectiveness of the bioencapsulation and the probiont was bioencapsulated in all cases in the first 3 h to a level of 10^2 cfu rotifer^{-1}. When the rotifers with the bacteria bioencapsulated were transferred to green-water tanks and kept in the conditions used in turbot larvae rearing, *Phaeobacter* 27-4 maintained in levels close to 10^2 CFU rotifer^{-1} for 48 h in the case of E24 and E3, and for 24 h in the case of E3+, a period of time sufficient to the larvae to graze on them and to incorporate the probiotic. The E24 protocol was selected for the simplicity of the procedure. DGGE fingerprints showed the incorporation of the probiotic and a temporal colonization of the rotifers. Predominant bands identified in the rotifers correspond to γ-proteobacteria as *Pseudoalteromonas*.

Keywords: Probiotic; *Phaeobacter* 27-4; Bioencapsulation; Rotifer; Larval rearing

1. Introduction

In the last years, efforts have been made to develop strategies for microbial control, to decrease the use of therapeutic chemicals and antibiotics (Cabello, 2006), towards a more environmentally friendly and sustainable aquaculture. Probiotics, defined by FAO/WHO (2001) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host", constitute a potential tool in the reduction of mortalities in the rearing of aquatic organisms (Gatesoupe, 1999; Verschuere et al., 2000; Vine et al., 2006; Kesarcodi-Watson et al., 2008).

In turbot larvae (*Psetta maxima*), the use of probiotics has been studied using commercially available or selected terrestrial lactic acid bacteria (Gatesoupe, 1991; Planas et al., 2004). A better strategy, that avoids the introduction of exotic bacteria to the system, is to select probiotic candidates among isolated strains from healthy turbot (Westerdahl et al., 1991) or hatchery facilities (Huys et al., 2001; Hjelm et al., 2004a). Generally, selection is based on the antagonistic effect to pathogenic bacteria which are responsible of high mortalities in turbot larvae such as *Listonella (Vibrio) anguillarum* or *Vibrio splendidus* (Toranzo et al., 1994; Thomson et al., 2005).

Members of the *Roseobacter* cluster (α-*Proteobacteria*) produce tropodithietic acid (TDA), compound that inhibits to different α and γ-Proteobacteria (Martens et al., 2007). The strain *Phaeobacter* 27-4, used in this study, was isolated from turbot larval rearing units by Hjelm et al. (2004a) and sequence analysis of 16S rRNA gene showed 99.1 % alignment with *Phaeobacter gallaeciensis* (Ruiz-Ponte et al., 1998). *Phaeobacter* 27-4 showed antagonism against *L. anguillarum* and *V. splendidus* (Hjelm et al., 2004a, b). Furthermore, Planas *et al.* (2006) demonstrated, in challenge trials with *L. anguillarum*, a probiotic *in vivo* effect of *Phaeobacter* 27-4, being not harmful to turbot larvae.

Intestinal microbiota of turbot larvae is strongly dependent on the bacteria present in live prey and, to a lesser extent, in the rearing water (Nicolas et al., 1989; Munro et al., 1994; Blanch et al., 1997; Reitan et al., 1998). The rotifer *Brachionus plicatilis* is widely used as live prey in turbot hatcheries and mass culture of rotifers conducts to a

high load and a variable bacterial microbiota (Verdonck et al., 1994) in their external surface (Munro et al., 1993) and digestive tract (Skjermo and Vadstein, 1993). This microbiota is dominated by strains with a low degree of specialization and high growth rates (Salvesen et al., 1999) that can be detrimental to turbot larvae (Pérez-Benavente and Gatesoupe, 1988; Verdonck et al., 1997). This fact was demonstrated by the increment in the survival of larvae fed with axenic rotifers (Munro et al., 1995).

So, the control of bacterial microbiota in live feed is an important issue (Planas and Cunha, 1999; Dhert et al., 2001; Skjermo and Vadstein, 1999) and treatments based on disinfection of rotifer eggs for the production of axenic cultures, treatment with hydrogen peroxide or ultraviolet radiation for partial decontamination. can be useful tools (Dhert et al., 2001). However, the elimination of bacteria from live prey implies the loss of a stable microbial balance, predominated by K-strategists, and may favour a more rapid colonization by opportunistic bacteria with high growth rates (*r*-strategists), once the rotifers are introduced into the larval rearing system. Replacement of the opportunistic bacteria by a preventive colonization with other non-aggressive bacteria with persistence in water or live food can be a good strategy to provide protection to the larvae (Makridis et al., 2000a; Martínez-Díaz et al., 2003).

Delivery of probiotic bacteria to live prey can not only serve as control agent of opportunistic or pathogenic bacteria but also be a vehicle for introducing probiotics to fish larvae (bioencapsulation) (Gatesoupe, 1994, 1999; Skjermo and Vadstein, 1999; Ringø and Birkbeck, 1999; Makridis et al., 2000b). Feeding larvae with rotifers enriched with *Phaeobacter* 27-4, parallel to fish pathogen *Listonella anguillarum* infection, in an experimental challenge model (Planas et al., 2005), brought the accumulated mortality to the level of control, demonstrating the effectiveness of bioencapsulation of the probiotic in rotifers (Planas et al., 2006).

In a normal practice, before delivery to fish larvae, rotifers are enriched with essential fatty acids, such as polyinsaturated fatty acids (PUFAs), feeding them with microalgae (e.g. *Tetraselmis* sp., *Isochrysis* sp., *Rhinomonas* sp., *Rhodomonas* sp.*)* (Dhert et al., 2001), The "green water" technique, which consists in the addition of microalgae to the rearing tanks, reduces the proliferation of opportunistic bacteria in the surface of turbot larvae (Salvesen et al., 1999). Algal cultures associate a specific bacterial population

(Schulze et al., 2006), which might influence bacteria number and composition in rotifers and fish larvae, and members of the *Roseobacter* clade have been frequently found associated to microalgae cultures (Sandaa et al., 2003; Hjelm et al, 2004b; Nicolas et al., 2004). Algae can also be a factor influencing the grazing of bacteria by rotifers (Nicolas et al., 1989). Consequently, interactions bacteria-microalgae must be considered in the maintenance of the probiont in the rearing tanks and in the bioencapsultion process.

Monitoring of bacteria introduced in the rearing systems and studying the modification of the associated bacterial microbiota, is an important aspect to understand the way of action of probiotic bacteria. Generally, introduced strains are analysed by culture-dependent methods (e.g. Martínez-Díaz et al., 2003; Planas et al., 2006) and only a few studies use complementary culture-independent techniques to screen the introduced strains, such as inmunocolony-blot (Makridis et al., 2000a, b), ELISA (Makridis et al., 2000a, b) or in situ hybridization (Macey and Coyne, 2005). Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA is a very useful technique for genetic fingerprinting of the bacterial community and to monitor changes in its composition (Muyzer, 1997). Furthermore, the excision, re-amplification and sequencing of the bands from the DGGE gels makes possible to identify the bacteria present. In aquaculture, DGGE has been applied to the study of bacterial microbiota in rotifers (Rombaut et al., 2001) and larval rearing systems (McIntosh et al., 2008). Recently, DGGE has been used to study the effect of immunostimulatory substances on fish gut microbiota (Liu et al., 2008) and the effect of feeding and the introduction of probiotic bacteria in rotifer culture (Qi et al., 2009).

The aims of the present work were i) to study the survival capability of the probiotic strain *Phaeobacter* 27-4 maintained in clear and green seawater, ii) to evaluate different protocols for the bioencapsulation of the strain *Phaeobacter* 27-4 into rotifers, iii) to evaluate the residence time of the strain once the rotifers were transferred to larval rearing conditions, and iv) to monitor introduced probiotic bacteria and characterize bacterial populations in water and rotifers applying a DGGE technique.

2. Materials and methods

2.1. Bacterial strains

Phaeobacter (formely *Roseobacter*, Martens et al. 2006) strain *27-4* was isolated from the tank walls of a turbot hatchery (Stolt Sea Farm, Merexo, Spain) in Galicia (Northwest Spain) and identified by Hjelm et al. (2004a). The strain was supplied by Lone Gram at DTU Aqua (Lyngby, Denmark). *Listonella anguillarum* strain 90-11-287 (serotype O1) and *Vibrio splendidus* DMC-1 were isolated from rainbow trout (Skov et al., 1995) and from a turbot rearing unit (Thompson et al., 2005), and kindly supplied by Lone Gram (DTU Aqua, Denmark) and Harry Birkbeck (University of Glasgow, United Kingdom), respectively. *Tenacibaculum maritimun*-like strain was isolated from diseased turbot in a fish farm in Galicia, and kindly supplied by Ana Riaza (Stolt Sea Farm, Merexo, Spain). Strains *Alteromonas macleodii* ALR3, *Kordia algicida* ALR4, *Tenacibaculum discolor* ALR5, *Ruegeria mobilis* ALR6 and *Flexibacter* sp. ALH7 were isolated from the culture systems at IIM-CSIC pilot-plant (Vigo, Spain). All the strains were kept at –80 °C in Tryptone Soy Broth (TSB, Oxoid CM129) (30 g l^{-1}) with glucose (5 g l^{-1}), skimmed milk (20 g l^{-1}) and glycerol (40 g l^{-1}).

2.2. Bacterial culture

Phaeobacter 27-4 was cultured according to Hjelm et al. (2004a). Briefly, bacteria were pre-cultured in 3-4 ml of Marine Broth (MB, Difco 2216) and incubated at 20 °C for three days in the dark and stagnant aerobic conditions. Culture (1 ml) was used to inoculate a 1-l flask with 100 ml of MB and cultured in the same conditions for two days. Bacterial concentration was verified by serial dilutions in seawater and plating on Marine Agar (MA, Difco 2216-212185). These conditions ensured a bacterial concentration of 5 x 10^8 to 1 x 10^9 CFU ml^{-1}. The appropiate volume of culture was added to the water tanks or to the rotifer enrichment tanks to give a initial concentration of 10^7 CFU ml^{-1}.

2.3. Experiment 1 - Maintenance of Phaeobacter 27-4 in water

Phaeobacter 27-4 survival and residence time in water, under the conditions used for turbot larval rearing, was investigated in 25-l cylindrical metacrylate tanks containing

15 l of aerated seawater (>90% oxygen saturation) at 18 ºC and 35 ppt. The light intensity at water surface was 3.5 µE . sec^{-1} . m^{-2} (day light provided by fluorescent lamps). Two conditions were assayed in duplicate: clear seawater (CW) and green seawater (GW), with the addition of 2 x 10^5 cells ml^{-1} of *Isochrysis galbana*. All the experimental tanks were inoculated with 10^7 CFU ml^{-1} of *Phaeobacter* 27-4.

2.4. Experiment 2A - Rotifer culture and bioencapsulation of bacteria

Cultured *Brachionus plicatilis* (200 rotifers ml^{-1}) were fed with baker's yeast (*Saccharomyces cerevisiae*) and subsequently enriched with *Isochrysis galbana* (2 x 10^6 cells ml^{-1}) for 24 h in 25-l tanks containing 10 l of aerated seawater (>90% oxygen saturation) at 23 ºC and light (daylight provided by fluorescent lamps). Three different ways of bioencapsulation of *Phaeobacter* 27-4 into rotifers were assayed in duplicate:

- E24: addition of *Phaeobacter* (10^7 CFU ml^{-1}) for 24 h in the enrichment with *I. galbana.*
- E3: addition of *Phaeobacter* (10^7 CFU ml^{-1}) during the last 3 h of the enrichment with *I. galbana*
- E3+: after the 24 h enrichment with *I. galbana*, the rotifers were filtered (30 µm Nylon mesh), washed and transferred (200 rotifers ml^{-1}) into 10-l tanks containing *Phaeobacter* (10^7 CFU ml^{-1}) in seawater and maintained for 3 h.

2.5. Experiment 2B - Residence of Phaeobacter 27-4 in rotifers

In all cases, after the bioencapsulation, the rotifers were filtered, washed with seawater, and transferred (5 rotifer ml^{-1}) to 25-l cylindrical metacrylate tanks containing 10 l of aerated seawater (>90% oxygen saturation) at 18 ºC and 2 x 10^5 cells ml^{-1} of *I. galbana*. The light intensity at water surface was 3.5 µE . sec^{-1} . m^{-2} (day light provided by fluorescent lamps). The organisms were maintained for 72 h under these culture conditions.

Experimental time, computed from the beginning of the enrichment process, was 96 h (E24, E3) or 99 h (E3+). A partial water exchange (30-40 %) was done at 72 h with the

addition of seawater and a volume of *I. galbana* culture (2 x 10^5 cells ml^{-1}). These conditions reproduced those used for turbot larval rearing.

2.6. Experiment 3. Bioencapsulation of Phaeobacter 27-4 in rotifers with the selected protocol

The selected protocol was used with three different batches of rotifers. Total bacteria and total *Vibrionaceae* in rotifer were determined at the end of the bioencasulation process.

2.7. Microbiological methods

Samples from water and rotifers were taken under aseptic conditions during the trials. In *Exp* 1, samples were taken from water at different times from 0 to 120 h. At the same time, the microbiota adhered to the wall of the tanks was sampled scraping daily the same area (daily colonization) or a different adjacent area (cumulated colonization). The sampling area (2 x 2 cm) was located at a medium level of the water column. At the end of the experiment (144 h) samples were taken from the aggregates that flocked at the bottom of the tanks, in an area of 4 x 4 cm. In *Exp* 2, 400 rotifers were filtered using a 30 µm Nylon mesh, washed with sterile seawater, collected in an Eppendorf tube and placed in ice for 30 min to facilitate rotifers decantation. Excess seawater was discarded and the final volume adjusted to 0.1 ml. Rotifers were then homogenised using an Eppendorf micropestle and the final volume adjusted to 0.5 ml with autoclaved seawater.

Homogenized samples were serially diluted in sterile seawater, plated on MA and incubated for 3 days at 20 ºC in the dark. Plates with 30 to 300 colonies were counted and predominant colonies were isolated. *Phaeobacter* 27-4 colonies were identified by their dark brown pigmentation and confirmed by absence of growth on Tryptone Soy Agar (TSA, Oxoid CM131) plates (Hjelm et al., 2004a). For the identification of *Vibrionaceae* bacteria, MA plates were replicated on Thiosulphate Citrate Bile Sucrose (TCBS, Cultimed 413817) plates (Planas et al., 2005, 2006), which were incubated for 24-48 h at 20 ºC.

2.8. In vitro antibacterial activity in a well diffusion assay

Phaeobacter 27-4 and *Roseobacter* sp. ALR6 were tested for its inhibitory activity against the fish pathogens *Listonella anguillarum* 90-11-287, *Vibrio splendidus* DMC-1 and *Tenacibaculum maritimum*-like strain, as well as other bacteria isolated from the culture systems in the present work: *Alteromonas macleodii* ALR3, *Kordia algicida* ALR4, *Tenacibaculum discolor* ALR5 and *Flexibacter* sp. ALH7.

All the strains were pre-cultured in 4 ml of Marine Broth (MB, Difco) and incubated at 20 ºC for 72 h in the dark and under static conditions. One ml of the pre-cultures were inoculated independently into 100 ml of MB and incubated, under the same conditions, 24 h for *L. anguillarum* and *V. splendidus* and 48 h the rest of the strains. *L. anguillarum* and *V. splendidus* were sub-cultured once more under the same conditions. One ml of the different cultures was centrifuged (5000 x g / 4 ºC / 5 min) and the resulting pellet re-suspended in sterile seawater to obtain an optical density at 600 nm of 0.5. Sixty microlitres of the resulting suspension of the target strains were inoculated into 100 ml of MA, previously autoclaved (121 ºC/15 min) and cooled down to 44.5 - 45 ºC. Then, inoculated agar was spread on Petri dishes. Once the inoculated agar had solidified, 6-mm diameter wells were punched and filled with 60 µl of bacterial cultures and supernatants of *Phaeobacter 27-4* or *Ruegeria mobilis* ALR6. Autoclaved MB was used as control. After 24-48-h incubation at 20 ºC in the dark, the diameter of clearance zone was measured. All trials were conducted in duplicate.

2.9. DNA extraction

In *Exp* 1, two samples of 1.5 ml of water from the tanks were centrifuged (13 000 x g, 5 min) and precipitates pooled in one tube for DNA extraction. At the end of the experiment (144 h), the aggregates settled in an area of 16 cm^2 at the bottom of the tank were harvested and approximately 50 ml of the clarified water was filtered through a cellulose acetate capsule filter with a pore size of 0,2 µm and 26 mm of diameter (Filter-Lab, Filtros Anoia, Barcelona, Spain). DNA extraction was conducted directly from the filters. In *Exp* 2, half of the volume of the homogenised rotifers (corresponding to 200 rotifers), was used for DNA extraction.

DNA was extracted by NucleoSpin Tissue Kit (Macherey-Nagel, GmbH and Co. KG, Düren, Germany), following manufacter's instructions, with a final volume of 35 µl. Total DNA extracted was quantified by UV spectrometry at 260 nm. For the extraction

from filters, the pre-lysis buffered solution was added to the filter capsule and then sealed and incubated at 56 ºC for 30 min. Subsequently, all the volume in the capsule was transferred to an Eppendorf tube and the filters were washed with the remaining pre-lysis buffered solution volume. The pooled volume was incubated at 56 ºC for 1.5 h and afterwards processed following the manufacter's protocol.

2.10. Polymerase Chain Reaction (PCR)

For DGGE analysis, two primers sets and conditions: (A) gc-338f and 518r (Muyzer et al., 1993), (B) gc-358 and 907rM (Muyzer et al., 1997) were assayed. The first one amplifies approximately 200 bp and the second 550 bp. Amplification was performed in a GeneAmp 2700 PCR System (Applied Biosystems) thermal cycler in the conditions previously described (Muyzer et al., 1993).

Aliquots (2.5 µl) of the amplification products were analyzed first by electrophoresis in 2% agarose gels and quantified using a Precision Molecular Mass Ruler (BioRad) marker.

2.11. Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR products were analyzed by DGGE using the Bio-Rad DCode apparatus following the procedure described by Muyzer et al. (1993). Fragments amplified with primer set A and with primer set B were loaded, respectively, on 8% or 6% (wt/vol) polyacrylamide gels in 1X TAE with 30 to 60% gradient urea-formamide (100% corresponded to 7 M urea and 40% [v/v] formamide) increasing in the direction of electrophoresis. For samples of water or rotifers 500 ng of PCR product were loaded on the gel. A control with 250 ng of PCR product from DNA extracted from pure cultures of *Phaeobacter* 27-4 was included. All parallel electrophoresis were performed at 60 °C. Gels were run for 10 min at 20 V and 3 h at 200 V, stained with ethidium bromide for 10 to 15 min and rinsed for 20 to 30 min in distilled water.

2.12. Sequencing of DNA from PCR fragments and bacterial isolates

DGGE bands were cut out with a sterile scalpel. Each fragment was washed with 200 µl of sterile water, and DNA eluted in 50 µl at 4 ºC during 48 h. Five microlitres of the

eluted DNA from each DGGE band was re-amplified by using the same conditions described above. The success of re-amplification and the purity of the bands were checked by loading 250 ng of PCR product on a new DGGE as described above, using as control the same sample from which bands were excised. PCR products that yielded a single band, which co-migrated with the original band, were then purified and sequenced.

DNA extracted from bacterial isolates was amplified by using the primers and conditions described in PCR-DGGE section and 100 ng of resultant PCR products were loaded in a DGGE as described above. The DNA from isolates showing a unique band in the corresponding DGGE pattern was then amplified with primers 27f and 907Mr in the conditions previously described (Ampe et al., 1999) and resultant PCR products were used for sequencing.

Twenty microlitres of PCR product were treated with 2 μl of Exonuclease I and 2 μl of Shrimp Alkaline Phosphatase (Amersham Pharmacia Biotech). The mixture was incubated at 37 °C for 30 min and then at 80 °C for another 15 min. Sequencing reactions were prepared with the ABI Prism dRhodamine Terminator cycle sequencing ready reaction kit (Applied Biosystems). To 4 μl of Terminator mix from the aforementioned kit, 90 to 200 ng of cleaned PCR product, 6.4 pmol of the corresponding primer and distilled water up to 10 μl were added. The components were mixed and the tube loaded in the thermal cycler. The conditions of the sequencing reaction were: 25 cycles with 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The extension products were purified using an ethanol/magnesium chloride precipitation procedure for the removal of the non-incorporated dye terminators. The pellet was dried at 30 °C with a centrifuge with a vacuum device and stored at -20 °C. Once the extension products were purified, electrophoresis was carried out in an ABI PRISM™ 310 DNA Sequencer (Applied Biosystems). Prior to sample loading, the pooled and dried reaction products were suspended in loading buffer (Applied Biosystems), containing five parts of deionized formamide to one part of 25 mM EDTA pH 8.0.

The collected data from both polynucleotide strand sequences were processed using the software BioEdit and CLUSTAL to align the sequences. The sequences were compared against nucleotide sequences in the GenBank of the National Center for Biotechnology Database (NCBI) using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was reconstructed by the neighbor-joining approach with Jukes Cantor

correction using MEGA4. The robustness of the tree topology was verified through calculating bootstrap values for the neighbor-joining tree and through comparison with the topology of a maximum likelihood tree, calculated by using the default settings.

2.13. Statistical analysis

Differences in bacteria bioencapsulation in rotifer by the different protocols were analysed using one-way analysis of variance (ANOVA).

3. Results

3.1. Specificity of primers for DGGE

Both primers sets, gc338f - 518r (Muyzer et al., 1993) and gc358f - 907rM (Muyzer et al., 1997) were tested with axenic cultures of *I. galbana* and *B. plicatilis*. The identification by sequencing of the resulting bands showed that, although the primer pairs are reported as specific for bacteria, both pair sets amplified the *I. galbana* plastid 16S rRNA gene and gc338f - 518r also amplified *B. plicatilis* 18S rRNA gene. This fact may interfere the cluster analysis of fingerprints but not the identification of bands and the study of evolving of bacterial populations. We decided to use gc-338f and 518r (Muyzer et al., 1993) because we got a higher number of bands on the fingerprints with a similar identification (data not shown). The limit of detection of the technique was verified with different quantities of bacteria (from 10^8 to 10^5 CFU) with or without addition of rotifers (400 rotifers). *Phaeobacter* 27-4 band was detectable in the DGGE, down to 10^5 CFU, either in presence or absence of rotifers.

3.2. Experiment 1 - Maintenance of Phaeobacter 27-4 in seawater

The survival of *Phaeobacter 27-4* was investigated in clear (CW) or green seawater (GW). The monitoring of the bacterial microbiota by plating (CFU ml^{-1} in MA) showed that both treatments performed similarly (Fig. 1) as the concentration of *Phaeobacter* 27-4 in the water was independent of the addition of *I. galbana*. In both cases, the initial concentration of 10^7 CFU ml^{-1} increased one log unit during the first 24 h, and

decreasing gradually during the following 48 h down to the initial concentration. From 72 h, *Phaeobacter* 27-4 in water decreased sharply and was not detectable at 96 h.

Profiling the bacterial microbiota by DGGE (Fig. 2) provided similar patterns in both CW and GW. The addition of the probiont into the water promoted a shift in the bacterial community with a predominance of *Phaeobacter* 27-4 band (band 1CW and 1GW), detectable until 48-72 h. At that time, the concentration of *Phaeobacter* 27-4 in water, estimated by plate counting, was of about 10^6 CFU ml^{-1}, which is near to the detection limit of the method (data not shown). *Phaeobacter* 27-4 in pure culture showed a second faint band (band 20CW), which appeared also in the samples of seawater, in which the probiont was present.

Some of the predominant bands in the gel could be excised and successfully re-amplified and sequenced. The closest identities are shown in Table 1. All fragment sequences corresponded to portions of bacterial 16S rDNA gene, except for bands 2GW and 11GW, which were only present in GW tanks and exhibited 99% homology with *Isochrysis* sp. plastid 16S rDNA.

The sequence of the band from 27-4 (band 1CW and 1GW) showed 100% similarity with *Phaeobacter* 27-4 (AJ536669). The sequence of the second faint band (band 20CW and 23 GW) corresponded with 98% similarity to the sequence of the *Phaeobacter* 27-4 (AJ536669). The alignment of both sequences showed a 2 bp gap.

Predominant bands sequences showed similarities with α– and γ-*Proteobacteria*, *Flavobacteria, Sphingobacteria* and *Bacteriodetes* classes (Table 1). Some bands that were present before the addition of the probiont (e.g. bands 5 to 6 and 13 to 14 in CW and bands 3 to 4 in GW) become less predominant and some as band regained intensity after the disappearance of *Phaeobacter 27-4* in the water (e.g. bands 18 and 19 in CW and bands 12 and 13 in GW). Gamma-protebacteria were predominant in CW, but less abundant in GW (Table 1). At the end of the experiment (72-120 h), other bacteria were identified, belonging to the *Flavobacteria* group, as *Flavobacterium* sp. (band 16CW), and *Gelidibacter* sp. (band 10GW) or a *Bacteriodetes* bacterium (band 14GW). Also, *Flexibacter* sp. (band 9GW) and a bacteria belonging to the genus *Roseobacter* (band 22GW) were present in green water.

Three main periods can be established from DGGE profiles:

- 0 to 24 h: *Phaeobacter* 27-4 band was predominant,

- 24 to 96 h: *Phaeobacter* 27-4 gradually disappeared and *Tenacibaculum discolor* became predominant (bands 10CW and 6GW).
- 96-120 h: profiles became more similar to the initial conditions (time 0, before the addition of 27-4), with predominance of γ-proteobacteria from *Oceanospirillaceae* family.

In each situation, CW or GW, cumulated and daily colonization, for both total bacteria and *Phaeobacter* 27-4, were similar (Fig. 1). The levels of *Phaeobacter* 27-4 were stable until 48 h and dropped afterwards. From then it was no longer detected in CW tank wall samples but it was in GW tank walls.

In the aggregates that flocked at the bottom of the tanks at the end of the experiment, total bacteria in CW and GW were 1.6×10^5 and 3.1×10^5 CFU mm^{-2}, respectively. In one replica of each of the treatments tanks, *Phaeobacter* 27-4 was detected in the aggregates at a concentration of 10^3 CFU mm^{-2}, although it was not detected in water samples. Therefore, it seems that *Phaeobacter* 27-4 can remain within the aggregate for at least 144 h.

DGGE profiles for bacterial microbiota at the end of the experiment (144 h) were different in samples of aggregates and water (Fig. 3). A clear band (band 1A) corresponding to *Phaeobacter* 27-4 (Table 2) appeared in profiles of aggregates in tank 1 CW and tanks 1 and 2 (more faintly) of GW. Bands corresponding to 27-4 were not detected in the water. The identification of predominant bands (Table 2) confirmed the presence of the strain with similarity to *Tenacibaculum discolor* (band 2A) in aggregates and water for both treatments (CW and GW). Similarly, the bands with homology to *Bacteriodetes* (band 6A), and to *Neptuniibacter* (band 15W) were also recognized in aggregates and water in both treatments (CW and GW). Band 4A, (uncultured γ-proteobacteria) only appeared in treatment CW, both in aggregates and water. The band corresponding to *Gelidibacter* sp. (band 8A) was detected only in the aggregates in CW and GW. A band corresponding to *Flavobacterium* sp. (band 9A) was only clearly detected in the aggregates of tank 2 CW. Band 17W with a sequence with homology to the genus *Roseobacter* sp. or *Rugeria* sp. was only predominant in the water of the GW tank. Finally, the band 13W was only detected in the water of one GW tanks and corresponded to *Isochrysis* sp. plastid 16S rDNA.

3.3. Exp 2A - Bioencapsulation of Phaeobacter 27-4 in rotifers

The levels of *Phaeobacter* 27-4 in rotifers at the end of the bioencapsulation did not show significant differences (ANOVA: p=0.271) among treatments E24, E3 and E3+ (Fig. 4). The concentration of the probiont was approximately (ca. 2.5 – 3.0 x 10^2 CFU rotifer^{-1}), representing 15% of total bacteria in rotifers. The introduction of the probiotic did not affect the survival and growth of the rotifer (data not shown).

As could be observed in treatment E24, rotifer incorporated quickly the probiont during the first 3 h, attaining a maximal concentration of 3.64 x 10^3 CFU rotifer^{-1} at 6 h, and decreasing slowly afterwards to final values of 2.54 x 10^2 CFU rotifer^{-1} at 24 h. The bioencapsulation of the probiont jointly with the algae from the beginning (E24), is more effective and promotes a higher level of incorporation (80% of total bacteria in 3-6 h) than in the other treatments in which rotifer were previously enriched with the algae (E3 and E3+). However, the presence of the algae did not seem to interfere with the incorporation of the probiont. Both, in presence (E3) or absence (E3+) of the algae the concentrations of 27-4 were similar.

3.4. Exp 2B - Residence of Phaeobacter 27-4 in rotifers

The profiles of disappearance of the probiont (Fig. 4), once transferred the rotifers to rearing tanks, were similar for E24 and E3. In both cases, the concentration of the probiont in the rotifer was maintained, with a slow decrease, during 48 h, and decreasing sharply afterwards until total disappearance at 96 h (72 h in the rearing tank). With treatment E3+, the permanence of *Phaeobacter* 27-4 in the rotifer was shorter, not being detectable at 72 h (48 h in the rearing tank).

In DGGE profiles of rotifers treated with the E24 protocol (Fig. 5), *Phaeobacter* 27-4 band (band1) was detectable during all the bioencapsulation process. Similarly, but less intense, *Phaeobacter* 27-4 band was detected in rotifers at the end of the bioencapsulation using the E3 and E3+ protocol at 24 and 27 h, respectively. In all cases, once the rotifers were transferred to the tanks and kept in the conditions of turbot larvae rearing, a faint band corresponding to the probiont was observed at 6 h. After 24 h, the band corresponding to *Phaeobacter* 27-4 was not detected.

Predominant bands from E24 and E3+ DGGE gels were isolated, re-amplified and sequenced. Their closest identities are shown in Table 3. With E24 protocol, bands $2E_{24}$, $3E_{24}$ and $4E_{24}$, appeared in the rotifers before the addition of the probiont (at t_0), during all the bioencapsulation process, and maintained in rotifers once transferred to the rearing tanks with green water. Band $5E_{24}$, appeared during the bioencapsulation process corresponded to the second band of *Phaeobacter* 27-4. Band $6E_{24}$, $7E_{24}$ and $8E_{24}$ were detectable at the end of the bioencapsulation and band $7E_{24}$ kept to be detectable in rotifers during their maintenance in the rearing tanks. During the maintenance band $10E_{24}$ identified as *Kordia algicida*, became predominant in the rotifers.

With the E3+ protocol, the rotifers were first kept for 24 h in the normal enrichment with *I. galbana* conditions, and subsequently collected, washed and included in the *Phaeobacter* 27-4 bacterial suspension. Therefore, the fingerprint at 24 h would correspond to the usual conditions, without addition of the probiotic. Similarly to the E24 trial at time 0, before the addition of the probiont, the same bands $2E_{3+}$, corresponding to *Pseudoalteromonas* and $3E_{3+}$, an uncultured not identified bacteria appeared, were less predominant in the presence of the probiont and reappeared during the maintenance (bands $12E_{3+}$ and $13E_{3+}$, respectively). Some bands, as band $6E_{3+}$ (not successfully identified) were present in rotifers during all the experiment and others as band $7E_{3+}$ and $8E_{3+}$ were not detectable in rotifers during the maintenance. As for bioencapsulation with the E24 protocol, *Kordia algicida* (band $15E_{3+}$) became predominant in the rotifers during the maintenance in the rearing tank. None of the identified bands corresponded with *Vibrionaceae*.

3.4. Isolation of predominant strains

In the bioencapsulation experiments, predominant colonies were isolated from MA plates, purified and identified by DNA extraction and sequencing (Table 4). Some of the isolates sequences clustered in the phylogenetic tree (Fig. 6) with the bands sequenced from the DDGE gels. A strain isolated from rotifers, with showed similarity to *Vibrio* sp. (ALR1), was not detected in the DGGE gels. Probably this bacterium was less predominant in rotifers but better cultured in MA. Opposite, some bands detected in the

DGGE gel could not be isolated from MA plates, some of them could be probably not cultivable in that medium.

3.5. In vitro antagonism of Roseobacter strains

Phaeobacter 27-4 and *Roseobacter* sp. ALR6 strain, isolated from the rotifers in this study, were assayed for their ability to inhibit the growth of the fish pathogens: *Listonella anguillarum* 90-11-287, *V. splendidus* DMC-1 and *T. maritimum*-like strain. Also, it was assayed with the bacteria isolated from the culture systems: *Alteromonas macleodii* ALR3·, *Kordia algicida* ALR4, *Tenacibaculum discolor* ALR5, and *Flexibacter* sp. ALH7 (Table 5). *Phaeobacter* 27-4 cultures showed antagonism against all bacteria tested except the isolate *Tenacibaculum* sp. The filtered supernatant only inhibited the growth of *L. anguillarum*, *V. splendidus* and *T. maritimum* (Table 5). *V. splendidus* was not totally inhibited but a reduced growth of the pathogen and a double halo was observed. This could be due to an initial inhibition of the growth of the pathogen that could be surpassed afterwards. ALR6 cultures and supernatants caused clearings in the plates of both *Vibrio* tested and also in *T. maritimum* cultures, but no double halo was observed.

3.6. Exp 3. Bioencapsulation of Phaeobacter 27-4 in rotifers with the selected protocol

The E24 protocol was selected because of a higher efficiency of bioencapsulation and residence time of the probiont, and also for the simplicity of the single-step procedure. E24 protocol was used in a new experiment, with three different batch of rotifers in three different days. Total *Vibrionaceae* in rotifer were determined by replica-plating MA plates in TCBS. The concentration of *Vibrionaceae* in rotifer control was 2.1×10^2 ($+/- 2.1 \times 10^0$) CFU rotifer^{-1}, the 14% of total bacteria in MA ($1.5 \times 10^3 +/- 2.1 \times 10^0$) CFU rotifer^{-1}. In the rotifer with *Phaeobacter* 27-4 bioencapsulated at 9.4×10^1 ($+/- 1.1 \times 10^0$) CFU rotifer^{-1}, *Vibrionaceae* were 4.2×10^1 ($+/- 7.5 \times 10^0$) CFU rotifer^{-1}, which corresponded with the 6% of the total bacteria in MA ($7.1 \times 10^2 +/- 1.1 \times 10^0$) CFU rotifer^{-1}. Therefore, the introduction of *Phaeobacter* 27-4 in the rotifers reduced in 42% the concentration of *Vibrionaceae* counted in TCBS.

4. Discussion

4.1. Maintenance of Phaeobacter 27-4 in water

Phaeobacter 27-4 is a marine bacterium that forms part of the *Roseobacter* clade which belongs to α-Proteobacteria class. *Roseobacter* clade dominates among marine algal-associated bacteria including algae blooms and algal cultures (Buchan et al., 2005). *Roseobacter* sp., have been found associated with cultures of *Isochrysis galbana* in hatcheries (Sandaa et al., 2003; Nicolas et al., 2004). *Phaeobacter* 27-4 was isolated from turbot rearing units in which rotifers were fed on the algae *Isochrysis* and *Rhinomonas*, although the bacterium was not isolated in the rotifers (Hjelm et al., 2004a).

Results showed that in non-axenic conditions, the maintenance of *Phaeobacter* 27-4 in water is not favoured by the presence of *I. galbana*. Algae-bacteria associations have a high specificity (Cole, 1982) and although it has been observed an increment in the growth rate of certain bacteria promoted by extracellular products of *I. galbana* (Avendaño and Riquelme, 1999), this seems not to be the case with *Phaeobacter* 27-4. It is known the role of the *Roseobacter* group in sulphur cycling in the sea and degradation of dimethylsulfoniopropionate (DMSP) produced by marine algae (Moran et al., 2003). Production of DMSP in several cultivated phytoplankton species showed to be highest in dinoflagelates than in prymnesiophytes (as *I. galbana*), or diatoms (Hatton and Wilson, 2007), which could be a selective fact to associated bacteria.

The presence of the algae in the green water did not affect either the number of total cultivable bacteria, as compared with clear water, although Salvesen et al. (1999) showed that the addition of *I. galbana*, both to filtered seawater and matured water, implied an increase of total bacteria. In our case, seawater is not axenic or matured and had an initial level of 10^7 CFU ml^{-1}. Differences can be also due to the way of culturing the microalgae, which have a high influence in bacterial load (Salvesen et al., 2000).

The results proved that *Phaeobacter* 27-4 inoculated at 10^7 CFU ml^{-1} can grow one Log and become predominant in the first 12 h, decreasing slightly afterwards, and maintaining levels around 10^6 CFU ml^{-1} until 72 h. It should be noted that the

experiments of maintenance in water were conducted without addition of any source of organic matter, and that during the usual procedure of rotifers enrichment or larvae rearing, the input of organic matter due to faeces promotes a longer permanence of *Phaeobacter* 27-4. In previous trials with larvae fed with rotifers loaded with *Phaeobacter* 27-4 (Planas et al., 2006), the probiont appeared in the water of the tanks and was detectable until 120 h. However, even in the low nutrient concentration tested in the present work, *Phaeobacter* 27-4 can remain in the water of the tanks for 24-48 h, a period that can be considered enough for rotifers to graze and incorporate the probiont.

Inhibitory activity of *Phaeobacter* 27-4 has been related with biofilm formation on surfaces (Bruhn et al., 2005, 2006) and *Roseobacter* species were predominant among the isolates with antagonistic activity in a one-year study in turbot larvae rearing systems and appeared most abundantly at the tank walls (Hjelm et al., 2004b). However, our results showed that *Phaeobacter* 27-4 did not colonise preferably the surface of the tanks. The concentration on the samples taken from the walls for daily or accumulated colonization was similar, and as observed in water, disappearing at 72 h, suggesting that the detected bacteria could come from the surrounding water when sampling. The only difference between green water and clear water was that *Phaeobacter* 27-4 was detectable in low levels at longer time in the walls of the tank with green water. However, biofilm formation has been observed when bacteria were cultured with Marine Broth on plastic (polystyrene) or metallic (stainless steel) surfaces (Bruhn et al., 2006). So, probably the absence of colonization in the present experiments was due to the lack of nutrients, which limited bacterial growth, or other factors, as competition with other bacteria.

Flocculation occurred in the tanks and at the end of the experiment (144 h) and *Phaeobacter* 27-4 was detected in the aggregates in one tank of each treatment, even when the probiont was not detectable in water samples. The presence of bacteria from the *Roseobacter* group in marine aggregates has been reported before (Wagner-Döbler and Bielb, 2006). This fact could also influence the maintenance of the probiont in the tanks in a long-term process, but will not be determinant for bioencapsulation in a short-term enrichment process, as the one proposed.

4.2. Bioencapsulation of Phaeobacter 27-4 in rotifers

Bioencapsulation of probiotic bacteria in rotifer cultures has proven to be a useful tool to introduce *Phaeobacter* 27-4 to larvae (Planas et al., 2006), but some aspects should be considered. Rotifers cultured in bacterial suspensions can accumulate large number of bacteria, and also digest part of the bioencapsulated bacteria (Makridis et al., 2000b). The presence of algae is another factor that can affect grazing of bacteria and modify the efficiency of bioencapsulation (Nicolas et al., 1989). To verify this point, three short-term enrichment and bioencapsulation protocols, with presence or absence of algae, were tested in the present work. The results showed that the presence of algae was not determinant in the effectiveness of the bioencapsulation, although treatments E24 and E3, in which algae were present, provided the best results. E24, besides a more effective initial incorporation, allows the bioencapsulation of the probiont simultaneously with all the enrichment period with microalgae, simplifying the procedure to a single step.

In this work, results showed that concentration of *Phaeobacter* 27-4 in the rotifers with E24 protocol, increases quickly during the first 3 h and, although there is a lost during the enrichment, bacteria was kept near to 3 x 10^2 CFU rotifer^{-1} for at least 24 h. These results are similar with those obtained by Martínez-Díaz et al. (2003) with strains of *Vibrio* and *Aeromonas* in monoaxenic rotifers, in which the number of bacteria in the rotifer increased during the first 1.5 to 3 h, maintaining afterwards levels near to10^3 CFU rotifer^{-1} during 6 to 24 h. It is important to keep the number of bacteria in rotifers in adequate levels, as turbot larvae showed a decrease in feeding rate when fed with rotifers with a high bacterial load (i.e. 5 x 10^4 CFU rotifer^{-1}, according to Pérez-Benavente and Gatesoupe, 1988; Nicolas et al., 1989).

Rotifers remain in the rearing water for several hours before they can be ingested, as in aquacultural practice fish larvae are fed with rotifers three times a day. Thus, the bioencapsulated bacteria should remain in the rotifers enough time to allow for the incorporation by the larvae fed on them. It is important to determine the rate of loss of the bioencapsulated bacteria, and the persistence of the modified bacterial composition (Makridis et al., 2000a). The selected E24 protocol, with a higher efficiency of bioencapsulation maintained the probiont in the rotifers at values close to 10^2 UFC

rotifer^{-1} for at least 48 h, a period of time enough for the larvae to graze it and incorporate the probiont.

4.3. Bacteria profiling by DGGE

DGGE was used to detect *Phaeobacter* 27-4 in the samples and to monitor and study the modification of the bacterial microbiota induced by the presence of the probiont. Assuming some general biases of the PCR based molecular techniques (Von Wintzingerode et al., 1997), some specific limitations of the use of DGGE in this case should be considered. The first is the amplification of Eukaryotic ribosomal DNA from rotifer or algae, a common fact that has been reported to cause interferences in the study of planktonic or benthic aggregated communities and biofilms (Lyautey el al., 2005). Secondly, a faint double band for the strain *Phaeobacter* 27-4 was observed, even in samples from pure cultures. Sequencing showed a difference of a 2 bp gap between them. Intra-species heterogeneity is a limitation for microbial community analysis, as bacteria may content more than one copy of the 16S rRNA gene, in some cases with heterogeneous sequences (Ueda et al., 1999). To solve both problems, sequence analysis should be done for the identification of the bands and the correct analysis of the fingerprints.

In water, the fingerprints showed a temporal predominance of the probiont for 48-72 hours independently of the presence of microalgae. Predominant groups of bacteria present in the samples belonged to *Flavobacteria*, γ-*Proteobacteria* and *Sphingobacteria*. Nicolas et al. (2004) found a large spectrum of culturable bacteria associated to *I. galbana* cultures in bivalve hatcheries. Bacteria included α- and γ-*Proteobacteria* and *Sphingobacteria*. α-*Proteobacteria* were predominant in all cases and in some hatcheries the *Rhodobacter* group (*Roseobacter* sp., *Ruegeria* sp.) was the most represented group. However, the authors observed a low percentage of recovery of cultivable bacteria associated to *I. galbana* from the different hatcheries due to the inability to re-grow many of the dominant isolates. In our results, the presence of the *I. galbana* promoted the appearance of two bands in the DGGE which were not detectable in clear water, which corresponded to *Flexibacter* sp. and *Roseobacter* sp. Both strains could be isolated from the MA plates (ALH7 and ALR6, respectively) and the

sequencing of a bigger fragment of the 16S rRNA gene, produced the same identification that the sequences of the DGGE bands. It could be hypothesized that those strains are associated with the algae culture. ALR6 was also isolated from rotifers enriched with *I. galbana*.

When *Phaeobacter* 27-4 decline in the water tanks, *Tenacibaculum discolor* (Piñeiro-Vidal et al., 2008) became predominant and *Flexibacter* sp. was only detectable at the end of the experiment. Some bacteria belonging or related to the genus *Tenacibaculum* or *Flexibacter* are responsible of flexibacteriosis in turbot (Piñeiro-Vidal et al., 2007), Dover sole (*Solea solea*), Senegalese sole (*Solea senegalensis*), Sea bass (*Dicentrarchus labrax*), and Atlantic salmon (*Salmo salar*) (Avendaño-Herrera et al., 2006) and *Tenacibaculum discolor* has been isolated from diseased Senegalense sole (Piñeiro-Vidal et al., 2008). *Phaeobacter* 27-4 showed antagonism against the isolate of *Flexibacter* sp. but not against the isolate of *Tenacibaculum discolor*, although did antagonize a pathogenic *T. maritimum*, isolated from diseased turbot in a fish farm in Galicia (NW of Spain). Thus, the predominance of *Tenacibaculum discolor* after the disappearance of 27-4 cannot be explained in terms of antagonism. However, it is interesting that both *Phaeobacter* 27-4 and ALR6 bacteria free culture supernatant were antagonist of the *T. maritimum* strain, demonstrating that both strains could be potentially used as a control agent against flexibacteriosis.

It has been reported that rotifer cultures are dominated by bacteria species with a low degree of specialization (Salvesen et al., 1999). Nicolas et al. (1989) observed that the bacteria associated to rotifer culture, identified by biochemical assays, were mainly *Pseudomonas*, *Vibrio* and *Aeromonas*, and to a lesser extent *Alteromonas* and *Acinetobacter*. In a two year survey and using both culture dependent and culture-independent approach (DGGE), McIntosh et al. (2008) observed a stable microbiota in rotifers, with predominance of putative *Arcobacter* sp. and unclassified *Rhodobacteraceae*, and other genera as *Roseobacter*, *Alteromonas* and *Vibrio* being detected at some times, during both years. In our bioencapsulation experiments, bacteria in rotifers showed a predominance of γ-Proteobacteria such as *Pseudoalteromonas*, which were not detected in clear or green water. Bands corresponding to *Pseudoalteromonas* sp. or *Neptuniibacter* sp., remained detectable in rotifers during bioencapsulation and maintenance. *Tenacibaculm discolor* was not detected in the

rotifers. Strains similar to *Alteromonas macleodii* and *Vibrio* sp. were not detected by DGGE, although were isolated from rotifer cultured samples. Probably, these bacteria were less predominant in the rotifers but better cultured in MA. Opposite, some bands detected in the DGGE corresponded to bacteria (e.g. *Pseudoalteromonas*) which could not be isolated from MA plates, indicating that some of them may not be cultivable in that medium. A band corresponding to *Kordia algicida* became predominant in rotifers once transferred to the rearing tanks. This fact could be explained by the disappearance of *Phaeobacter* 27-4 which showed antagonism for that strain. Furthermore, bioencapsulation of *Phaeobacter* 27-4 reduced (42%) the concentration of *Vibrionaceae* in rotifers, demonstrating not only to be a way of introduction of the probiont to the larvae but also an effective tool for *Vibrionaceae* control in rotifers.

It can be concluded that the proposed bioencapsulation protocol permits to incorporate effectively the probiotic bacteria *Phaeobacter* 27-4 in rotifers, in adequate levels and enough time to get the probiont introduced to turbot larvae. By using PCR-DGGE coupled to sequence analysis of the isolated bands, the evolving of bacterial microbiota in water or in rotifer can be analysed and the modification promoted by the use of the probiont studied, verifying in the obtained fingerprints a temporal predominance of the probiont. In some cases, the shift in bacterial composition was explained based on antagonism of the probiont on isolated bacteria. DGGE as other culture-independent methods, permits the study of microbial communities which cannot be cultured in laboratory, which are estimated to be about 99% of the bacteria (Amann et al., 1995). Although in aquaculture systems, rich in nutrients, the percentage of cultivable bacteria may be high, culture based methods are labour and time consuming and not suitable to monitor the introduced strains and the induced modification of bacteria microbiota. DGGE and sequencing demonstrates to be a useful tool to monitor changes in bacteria communities and to identify bacterial groups in the use of probiotics in aquaculture.

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Figure captions

Fig. 1. Evolution of total bacteria and introduced *Phaeobacter* 27-4 in clear and green water, with *Isochrysis galbana* 2 x 10^5 cells ml^{-1}. In **A** and **B**: total bacteria (○) and *Phaeobacter* 27-4 (●) suspended in the water. In **C** and **D**: total bacteria (daily, ○ or cumulated, ◊) and *Phaeobacter* 27-4 (daily, ● or cumulated, ♦) attached to the walls of the tanks. Data represent mean ± standard deviation

Fig. 2. DGGE profiles of the bacterial communities in clear water and green water (with *Isochrysis galbana* 2 x 10^5 ml^{-1}) with addition of *Phaeobacter* 27-4 (10^7 CFU ml^{-1}) maintained under turbot larvae rearing conditions. Numbered bands were excised for sequencing and the similarity to other sequences is indicated in Table 1. Highlighted, the bands corresponding to *Phaeobacter* 27-4.

Fig. 3. DGGE profiles of the bacterial communities in the aggregates (A) and in the water (W) in tanks with clear water (CW) and green water (GW) (with *Isochrysis galbana* 2 x 10^5 ml^{-1}) with addition of *Phaeobacter* 27-4 (10^7 CFU ml^{-1}) maintained under turbot larvae rearing conditions for 144 h. Numbered bands were excised for sequencing and the similarity to other sequences is indicated in Table 2. Highlighted, the bands corresponding to *Phaeobacter* 27-4.

Fig. 4. Evolution of total bacteria (○) and *Phaeobacter* 27-4 (●) in rotifers. Grey zones represent bioencapsulation period of the rotifers (200 rotifer ml^{-1}) with *Phaeobacter* 27-4 (10^7 CFU ml^{-1}). *E24*: for 24 h in the enrichment with *I. galbana; E3*: during the last 3 h of the enrichment with *I. galbana*; *E3+*: after the 24 h enrichment with *I. galbana*, rotifers were filtered, washed and transferred tanks containing *Phaeobacter* 27-4 in seawater and maintained for 3 h. In all cases, after bioencapsulation, the rotifers were collected, washed and transferred (5 rotifer ml^{-1}) to tanks with green water (*I. galbana* 2 x 10^5 ml^{-1}).

Fig. 5. DGGE profiles of the bacterial communities in rotifers, during the bioencapsulation of *Phaeobacter* 27-4 using different protocols (see text), transferred afterwards to tanks with green water and maintained under turbot larvae rearing

conditions. Numbered bands were excised for sequencing and the similarity to other sequences is indicated in Table 3. Highlighted, the bands corresponding to *Phaeobacter* 27-4.

Fig. 6. Phylogenetic tree based on partial (about 150 bp) 16S rDNA sequences from excised DGGE bands and from MA isolates, related to different bacterial groups. The tree was constructed with the neighbour-joining method of the MEGA program package, with the Jukes-Cantor correction. *Thermogota maritima* DSM 3109T was used as outgroup. The scale bar corresponds to 0,1 substitutions per nucleotide.

Figure(s)

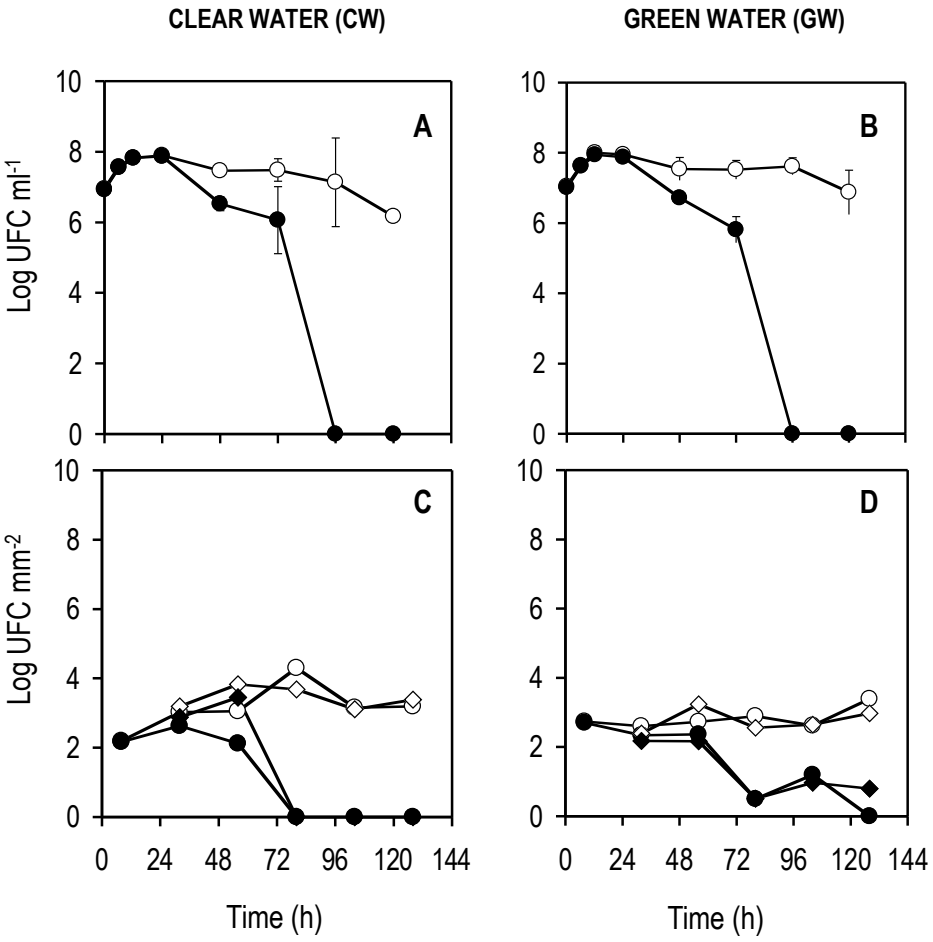

Figure 1

Figure(s)

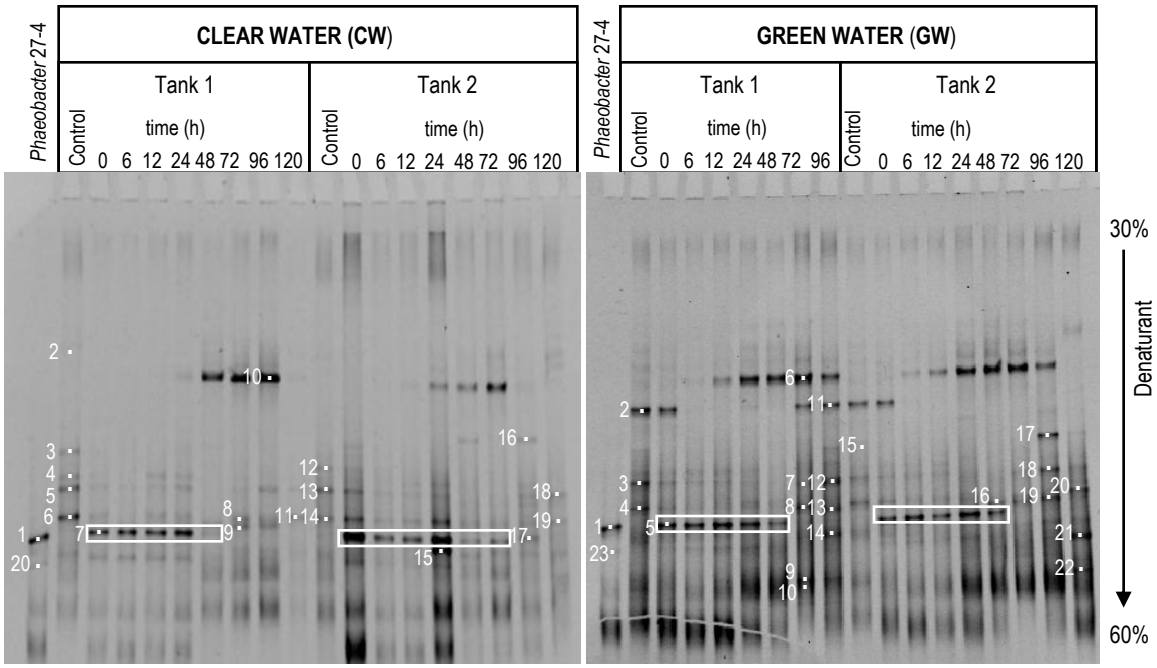

Figure 2

Figure(s)

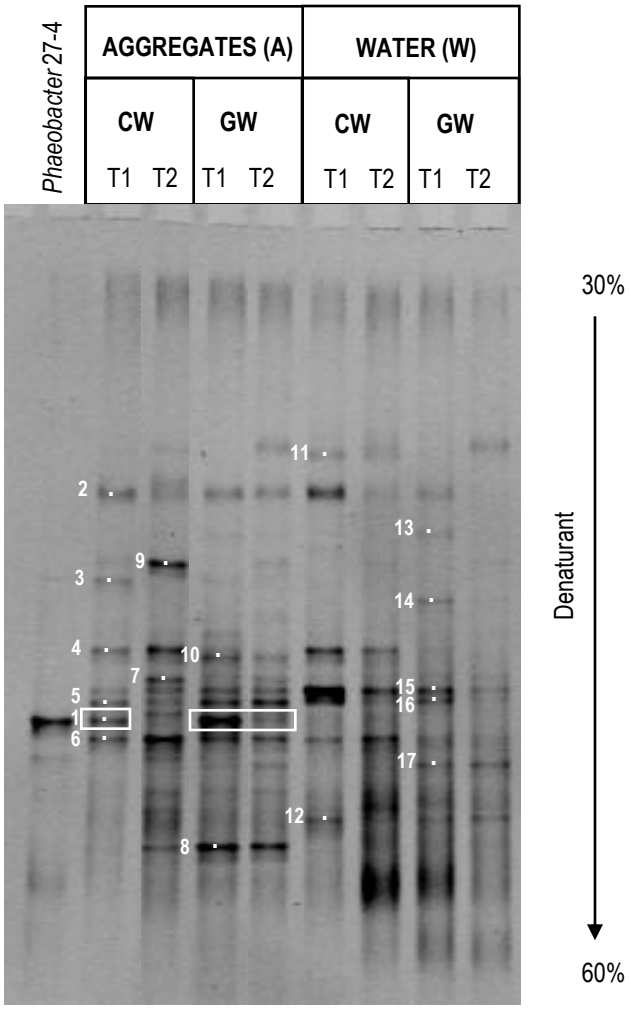

Figure 3

Figure(s)

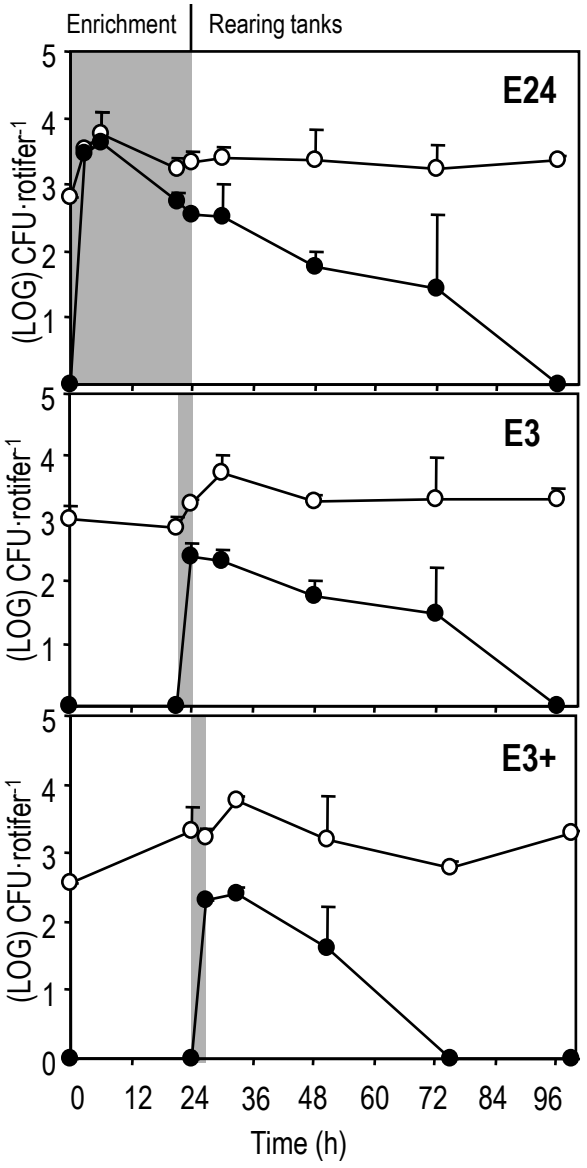

Figure 4

Figure(s)

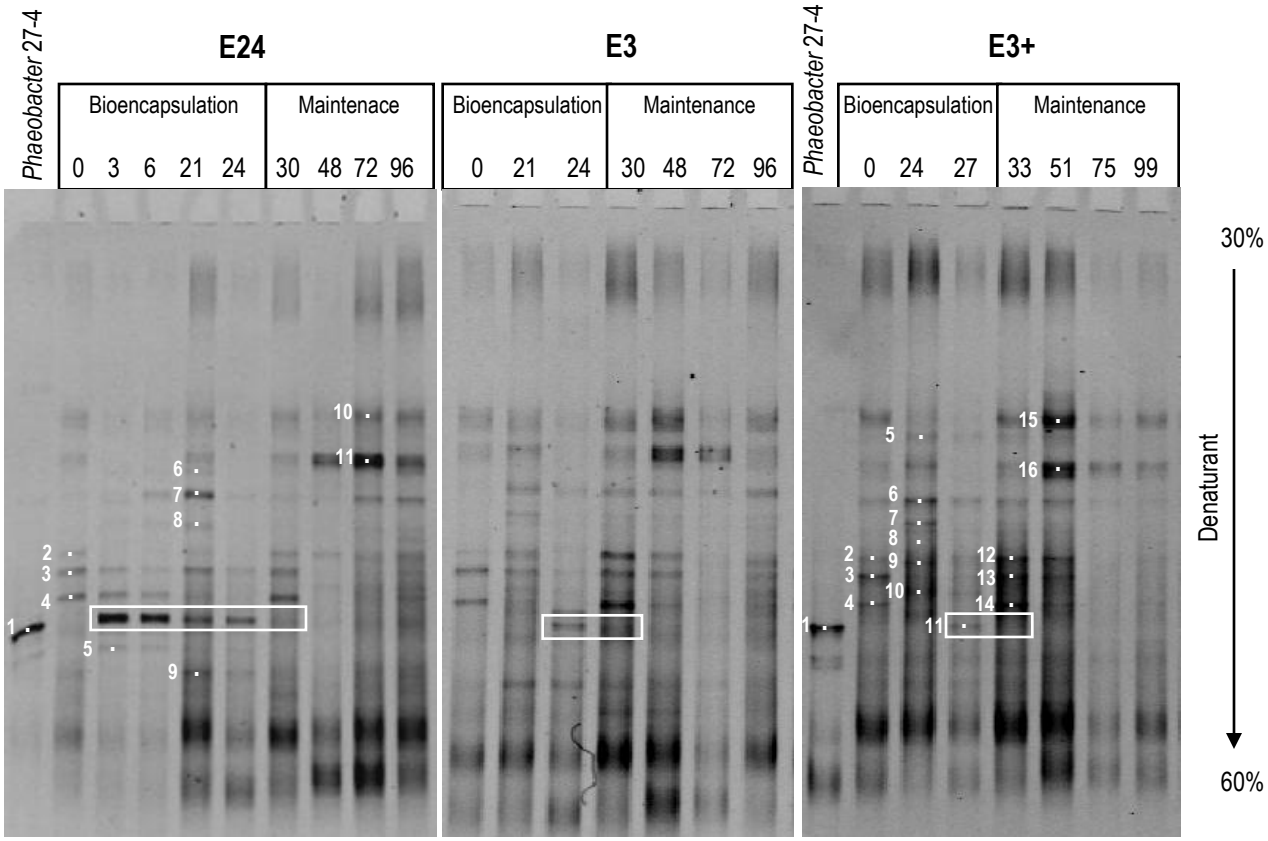

Figure 5

Figure(s)

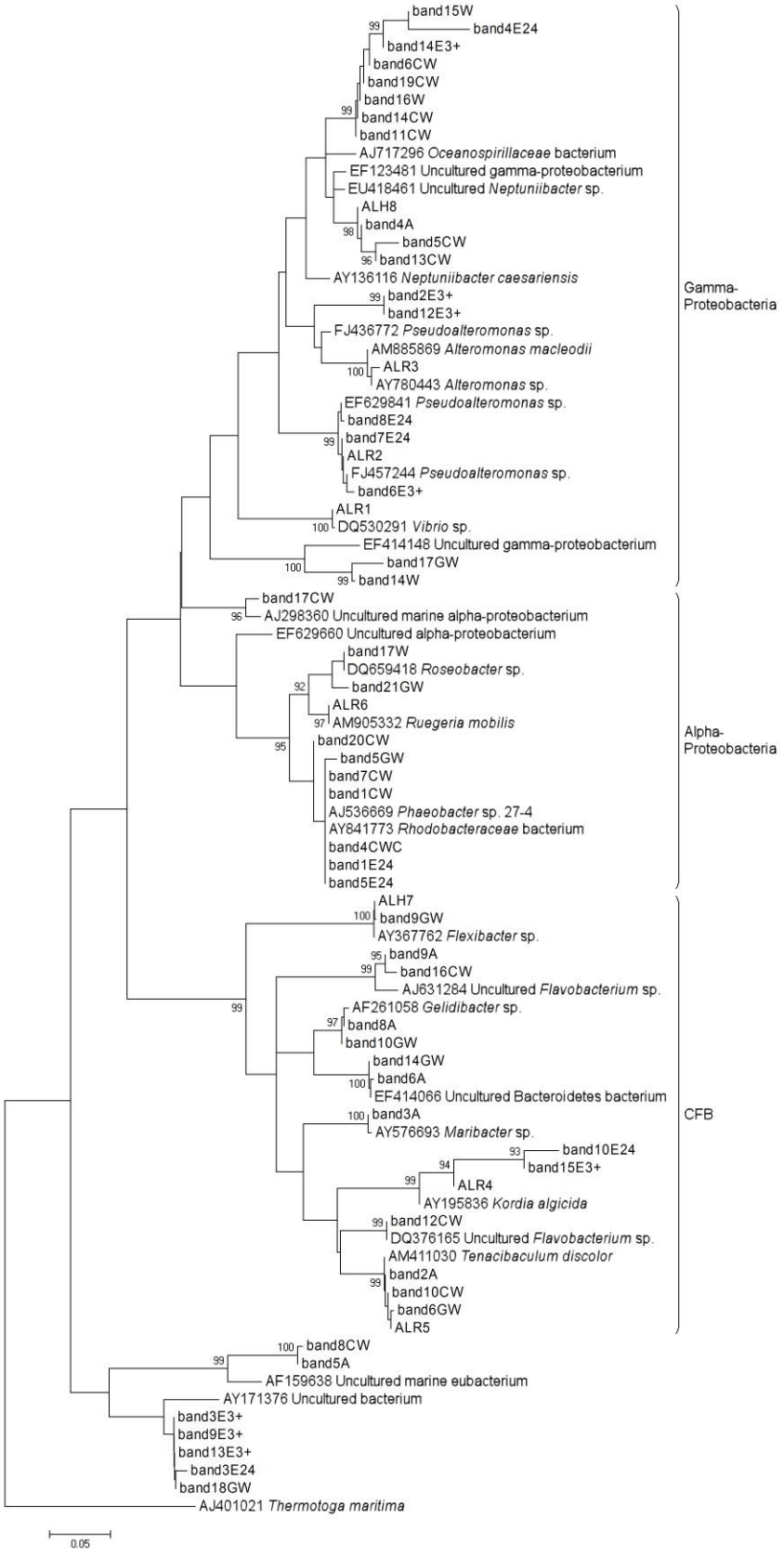

Figure 6

Tables

Table 1 Sequence similarities of the excised bands that appear in Figure 2. CW: clear water; GW: green water.

Band		Closest relative		
Number	% Similarity	Species	Accession number	Taxon (phylum or class)
1CW	100 (135/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
2CW	ni			
3CW	ni			
4CW	100 (135/135)	*Rhodobacteraceae*	AY841773	α-proteobacteria
5CW	92 (72/78)	*Neptuniibacter caesariensis*	AY136116	γ-proteobacteria
6CW	100 (156/156)	*Tenacibaculum discolor*	AM411030	Flavobacteria
7CW	100 (135/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
8CW	92 (144/156)	Uncultured marine bacterium	AF159638	bacteria
9CW	ni			
10CW	100 (151/151)	*Tenacibaculum discolor*	AM411030	Flavobacteria
11CW	95 (152/160)	Uncultured *Neptuniibacter* sp.	EU418461	γ-proteobacteria
12CW	100 (155/155)	Uncultured *Flavobacterium* sp.	DQ376165	Flavobacteria
13CW	97 (156/160)	*Oceanospirillum* sp.	AY136116	γ-proteobacteria
14CW	95 (152/160)	Uncultured *Neptuniibacter* sp.	EU418461	γ-proteobacteria
15CW	ni			
16CW	96 (149/155)	*Flavobaacterium* sp.	AJ631284	Flavobacteria
17CW	97 (132/135)	Uncultured marine α-*proteobacterium*	AJ298360	α-proteobacteria
18CW	ni			
19CW	95 (152/160)	Uncultured *Neptuniibacter* sp.	EU418461	γ-proteobacteria
20CW	98 (119/121)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
1GW	100 (135/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
2GW	99 (155/156)	*Isochysis* sp. plastid 16S rRNA gene	X75518	Eukaryota
3GW	100 (135/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
4GW	ni			
5GW	99 (134/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
6GW	100 (176/176)	Uncultured Bacteroidetes bacterium	EF414066	Bacteriodetes
7GW	ni			
8GW	ni			
9GW	100 (155/155)	*Flexibacter* sp.	AY367762	Sphingobacteria
10GW	100 (155/155)	*Gelidibacter* sp.	AF261058	Flavobacteria
11GW	99 (136/137)	*Isochysis* sp. plastid 16S rRNA gene	X75518	Eukaryota
12GW	ni			
13GW	ni			
14GW	100 (176/176)	Uncultured Bacteroidetes bacterium	EF414066	Bacteriodetes
15GW	ni			
16GW	ni			
17GW	94 (135/143)	Uncultured gamma proteobacterium	EF414148	γ-proteobacteria
18GW	93% (161/172)	Uncultured bacterium	AY171376	Bacteria
19GW	ni			
20GW	ni			
21GW	96 (130/135)	*Roseobacter* sp.	DQ659418	α-proteobacteria
22GW	ni			
23GW	98 (119/121)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria

Sequences were aligned to their closest relatives in public databases by using the BLAST 2.2.15

Table 2 Sequence similarities of the excised bands that appear in Figure 3. A: agregate; W: water.

Band		Closest relative		
Number	% Similarity	Species	Accession number	Taxon (phylum or class)
1A	100 (135/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
2A	100 (155/155)	*Tenacibaculum discolor*	AM411030	Flavobacteria
3A	99 (154/155)	*Maribacter* sp.	AY576693	Flavobacteria
4A	95 (158/165)	Uncultured gamma proteobacterium	EF630112	γ-proteobacteria
5A	93 (151/161)	Uncultured marine bacterium	AF159638	bacteria
6A	100 (150/150)	Uncultured Bacteroidetes bacterium	EF414066	Bacteriodetes
7A	ni			
8A	100 (155/155)	*Gelidibacter* sp.	AF261058	Flavobacteria
9A	97 (151/155)	*Flavobaacterium* sp.	AJ631284	Flavobacteria
10A	ni			
11W	ni			
12W	ni			
13W	97 (133/137)	*Isochysis* sp. plastid 16S rRNA gene	X75518.1	Eukaryota
14W	94 (153/162)	Uncultured gamma proteobacterium	EF414148	γ-proteobacteria
15W	96 (150/155)	Uncultured *Neptuniibacter* sp.	EU418461	γ-proteobacteria
16W	96 (157/162)	Uncultured *Neptuniibacter* sp.	EU418461	γ-proteobacteria
17W	100 (135/135)	*Roseobacter* sp.	DQ659418	α-proteobacteria

Sequences were aligned to their closest relatives in public databases by using the BLAST 2.2.15

Table 3 Sequence similarities of the excised bands that appear in Figure 5. E_{24} bands correspond to the samples of rotifers from bioencapsulation protocol E24, E_{3+} bands correspond to the samples of rotifers from bioencapsulation protocol E3+.

Band		Closest relative		
Number	% Similarity	Species	Accession number	Taxon (phylum or class)
1 E_{24}	100 (135/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
2 E_{24}	ni			
3 E_{24}	94 (159/169)	Uncultured bacterium	AY171376	
4 E_{24}	ni			
5 E_{24}	100 (135/135)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
6 E_{24}	ni			
7 E_{24}	98 (150/153)	*Pseudoalteromonas* sp.	EF629841	γ-proteobacteria
8 E_{24}	98 (150/153)	*Pseudoalteromonas* sp.	EF629841	γ-proteobacteria
9 E_{24}	ni			
10 E_{24}	96 (96/100)	*Kordia algicida*	AY195836	Flavobacteria
11 E_{24}	98 (160/162)	*Brachionus plicatilis* 18s ribosomal RNA gene	AY218119.1	Eukaryota
1 E_{3+}	100 (136/136)	*Phaeobacter* sp. 27-4	AJ536669	α-proteobacteria
2 E_{3+}	92 (130/140)	*Pseudoalteromonas* sp.	FJ436772	γ-proteobacteria
3 E_{3+}	94 (129/137)	Uncultured bacterium	AY171376	Bacteria
4 E_{3+}	ni			
5 E_{3+}	ni			
6 E_{3+}	100 (151/151)	*Pseudoalteromonas* sp.	FJ457244	γ-proteobacteria
7 E_{3+}	ni			
8 E_{3+}	ni			
9 E_{3+}	94 (129/137)	Uncultured bacterium	AY171376	Bacteria
10 E_{3+}	ni			
11 E_{3+}	ni			
12 E_{3+}	92 (130/140)	*Pseudoalteromonas* sp.	FJ436772	γ-proteobacteria
13 E_{3+}	94 (129/137)	Uncultured bacterium	AY171376	Bacteria
14 E_{3+}	95 (151/156)	Uncultured *Neptuniibacter* sp.	EU418461	γ-proteobacteria
15 E_{3+}	98 (148/151)	*Kordia algicida*	AY195836	Flavobacteria
16 E_{3+}	100 (136/136)	*Brachionus plicatilis* 18s ribosomal RNA gene	AY218119	Eukaryota

Sequences were aligned to their closest relatives in public databases by using the BLAST 2.2.15

Table 4 Sequence similarities of the predominant strains isolated in MA.

Band		Closest relative		
Number	% Similarity	Species	Accession number	Taxon (phylum or class)
ALR1	99 (570/573)	*Vibrio* sp.	DQ530291.1	γ-proteobacteria
ALR2	100 (525/525)	*Pseudoalteromonas* sp.	EU246846.1	γ-proteobacteria
ALR3	99 (506/508)	*Alteromonas macleodii*	AM885869.1	γ-proteobacteria
ALR4	98 (521/527)	*Kordia algicida*	AY195836	Sphingobacteria
ALR5	100 (524/524)	*Tenacibaculum discolor*	AM411030.2	Flavobacteria
ALR6	100 (520/520)	*Ruegeria mobilis*	AM905332.1	α-proteobacteria
ALH7	99 (452/453)	*Flexibacter* sp.	AY367762.1	Sphingobacteria
ALH8	96 (157/162)	*Neptuniibacter caesariensis*	AY136116	γ-proteobacteria

Sequences were aligned to their closest relatives in public databases by using the BLAST 2.2.15

Table 5 Antagonistic activity of *Phaeobacter* 27-4 and *Ruegeria mobilis* ALR6 in the well diffusion agar assay. The results are mean and standard deviation from five replicates. In brackets, reduced growth.

	Halo diameter (cm)			
	Phaeobacter 27-4		*Ruegeria mobilis* ALR6	
Target strain	Culture[a]	Supernatant[b]	Culture	Supernatant
Alteromonas macleodii ALR3	1.20 ± 0.07	ND	ND	ND
Kordia algicida ALR4	1.62 ± 0.11	ND	ND	ND
Flexibacter sp. ALH7	1.24 ± 0.08	ND	ND	ND
Tenacibaculum discolor ALR5	ND	ND	ND	ND
Tenacibaculum maritimum-like strain	1.72 ± 0.13	0.75 ± 0.07	1.00 ± 0.08	0.78 ± 0.04
Listonella anguillarum 90-11-287	1.86 ± 0.05	1.36 ± 0.10	1.50 ± 0.13	1.23 ± 0.12
Vibrio splendidus DMC-1	(1.6 ± 0.00)	(1.40 ± 0.00)	1.25 ± 0.06	1.00 ± 0.18

[a]Cells and supernatant; [b]Filtered supernatant; ND: Clearing not detected.