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Water quality and microbial community structure in juvenile Atlantic cod (Gadus morhua L.) cultures ☆

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Abstract

The effect of water treatment and flow rate on young Atlantic cod juveniles was investigated in a 36-days experiment. Four different flow rates (10, 20, 40, and 70 times the effective tank volume per day) were set up in triplicate tanks within each of three rigs with recirculated, UV-radiated, and untreated water, respectively. Each of the 36 tanks was stocked with 200 weaned cod juveniles at a mean weight of 0.048g. Fish mortality was recorded daily in all tanks, and growth (wet weight) was determined at the end of the experiment. The microflora in the rearing water was investigated by means of PCR-DGGE and flow cytometry. Observed mortality was significantly higher at low flow rates while otherwise unexplained mortality (presumed to be due to cannibalism) was lowest in the recirculation system. No correlation was found between survival and growth. Growth was significantly affected by both water exchange rate and treatment, as the juveniles from high flow rates and the UV-treatment showed elevated growth rates. Both growth and survival scaled in accordance with metabolic

☆ Runar Thyrhaug passed away January 7, 2011. The authors wish to dedicate this paper to his memory.

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factors like oxygen saturation and unionized ammonia. Bacterial concentrations increased in all tanks and treatment from the beginning of the experiment to the end. The UV-treated and untreated water started at typical seawater concentrations (0.5-1 x10^6 mL^{-1}) and increased five to tenfold during the experiment. The recycled water tanks started with bacterial concentrations 2-5 times higher than the UV- and untreated experiments at the time of fish transfer, and ended up with 10 times higher concentrations in the end. Cluster analysis of the DGGE profiles separated the recirculation tanks, including the respective inlet water, from the flow-through systems, with one exception (the highest flow rate). Eighty-five % of the sequences clustered within the Gammaproteobacteria, further divided into four distinct clusters. One of the clusters was only detected in the recirculation system, and showed highest affiliation to bacteria belonging to the Alteromonas/Pseudoalteromonas genera. In contrast, bacteria belonging to the family Vibrionaceae were detected in the flow-through systems.

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Keywords: Recirculation; Water treatment; Water flow rate; Juvenile production; Cannibalism; Acidification; Vibrio; Pseudoalteromonas; Alteromonas; DGGE; Flow cytometry

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1. Introduction

The commercial production of Atlantic cod (Gadus morhua L.) is increasing, reaching 19712 metric tonnes in Norway in 2009 (www.fiskeridir.no). In order to sustain a viable and increasing cod production, juveniles have to be produced at predictable quantities under controlled conditions. However, variable survival and quality during cultivation of the early life stages are commonly experienced. This brings challenges to the establishment of a stable supply of cod juveniles. In this respect, water quality is of particular interest. Water quality may affect survival, growth, and quality of the fish. Two major components of water quality are flow rate and water treatment, which may affect both microbial conditions and physical and metabolic parameters like oxygen, ammonia, carbon dioxide, and pH.

As the cod larvae metamorphoses, and proceed through the juvenile stages, biomass and organic load increase in the rearing tanks. High concentrations of nutrients and high densities of fish larvae provide favourable conditions for opportunistic pathogenic bacteria (Vadstein et al., 2004). Usually, this is compensated by increasing the water exchange rate, with sufficient oxygen levels as the control parameter. Sometimes biomass density exceeds the possibilities of keeping oxygen saturation at safe levels, and aeration or oxygenation has to be applied. Flow rate may control not only the physical parameters in the tanks, but also microbial growth. Increasing flow rate will have a dilution effect and aid the removal of organic substrates generated by the larval rearing, as well as bacterial biomass. More knowledge is needed on how water exchange rate may affect microbial conditions, fish growth, and survival in relation to biomass density in fish tanks.

Water treatment on the other hand have the possibility to affect microbial composition directly by killing or enhancing selection of potential opportunistic fish pathogens, or more beneficially, providing conditions for non-opportunistic and harmless bacteria (Vadstein et al. 2004). The differentiation between opportunistic and non-opportunistic bacteria might also be explained as the division between r-strategic and K-strategic bacteria, which relates to the selection of life strategies promoting success in particular environments (Andrews and Harris, 1986). In this sense water treatment should aim at selecting for K-strategic non-opportunistic species and avoid dominance of r-strategic and potential pathogenic opportunists. Different water treatment systems in marine fish rearing include microbial maturation of inlet water, UV-radiation or ozonation, and recirculation systems. Microbial maturation of water by

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running the water through a maturation unit is shown to select for a non-opportunistic bacterial flora and to enhance larval growth and survival (Salvesen et al., 1999; Skjermo et al., 1997). Similarly, Verner-Jeffreys et al. (2004) reported increased growth and survival of halibut larvae in a recirculation system.

Finding the optimal conditions of flow rate and water treatment may enhance juvenile production and improve fish welfare. The present work therefore presents results on growth, survival, and microbial conditions for cod during early juvenile stages, reared with three different water treatments, each with four different flow rates.

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2. Materials and methods

2.1. Biological material

The experiment was carried out over 36 days from 10 June to 16 July 2004 at Institute of Marine Research (IMR), Austevoll Research Station, Norway. Cod eggs were collected from communal spring-spawning broodstock fish, and incubated in 70 l black polyethylene incubators, modified from van der Meeren and Lønøy (1998) with submerged water inlet and airflow at surface. Newly weaned cod juveniles were obtained from larval rearing after a protocol described in van der Meeren et al. (2007), except for switching live Isochrysis sp. algae with Nannochloropsis sp. algal paste (Reed Mariculture Inc., Campbell, CA).

2.2. Experimental setup and sampling

A 36-tank experiment was conducted where each of the tanks was equipped with a separate water inlet with a high-grade flow meter. The tanks were organized in three rigs each containing twelve tank units. The water used was from 160m depth in the fjord and sand-filtered. Each of the three rigs received a different water quality, achieved by treatment in a recirculation system, by UV-radiation and no treatment as a control. All the three water qualities were temperature adjusted to 12°C and aerated before use.

The recirculation unit was a TMC 5000 Marine System (Tropical Marine Centre, London, UK), containing a 750 litre pallet reservoir with GRP support grid, filtration circulation

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pump, mechanical bag filter unit, fluidised sand filter unit with 15 kg oolitic coral sand, trickling bio-tower with side wall flow-deflector rings and 70 litres if TMC Bio-Rings random fill media (210 m^2/m^3), conditioner (Teco CA2000) for maintaining stable temperature, and protein skimmer complete with venture pump and internal and external foam cup wash kits. The protein skimmer was used in conjunction with a Sander A1000 Ozoniser (Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) controlled by a Burkert ORP 8206 transmitter with a PFGK-gel-2A/A redox sensor (Christian Bürkert GmbH, Ingelfingen, Germany). The redox potential was kept between 320 and 340 mV, and pure oxygen was used to both facilitate ozone production and maintain oxygen levels at the lowest flow rate in the recirculation unit. Approximately 5% of the water in the recirculation rig was removed daily during tending of the tanks, and recirculation rate was therefore ca 95%. To allow establishment of sufficient biofilm activity in the biofilters at the low temperature of 12°C, the recirculation unit was started approximately 16 weeks prior to start of the experiment. During this period the recirculation unit with tanks, a total of 1.3 m^3, was constantly conditioned by feeding a daily amount of 1.7 g NH_4Cl by an Iwaki EH/S membrane pump (Iwaki, Tokyo, Japan), corresponding to the expected daily ammonia production from cod juveniles in the recirculation rig at experimental start-up, as calculated from Finn et al. (2002). During the experiment, $NaHCO_3$ was used to counteract the reduction in pH appearing in the recirculation system.

The UV-treatment was made by a TMC P8-440W UV unit (Tropical Marine Centre, London, UK), coupled in line with the water supply of this rig. The TMC P8-440W consisted of eight tubes each with a 55W UV lamp inside a quartz glass sleeve, giving off 18W UVc radiation at 253.7 nm. During treatment, the water had to pass through two such tubes. According to manufacturer, the complete unit has a maximum capacity of 265 l/min with was far above what was actually used (10.2 l/min).

Within each water treatment (rig), four different flow regimes were applied in triplicate tanks. The water exchange rate was 10, 20, 40, and 70 times (denoted 10X, 20X, 40X, and 70X) of the effective tank volume (33 litres) per day. Each tank was stocked with 200 weaned cod juveniles (on 10 June, 61 day after hatching) at a mean weight of 0.048g. The juveniles were gently caught in the rearing tank with a 1.5 litre beaker and carefully transferred and counted when released to the experimental tanks. The fish were fed in excess with a commercial formulated feed (AgloNorse: Trofi AS, Tromsø, Norway) in a 24 h cycle, using belt feeders.

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Light intensity was adjusted to 20µW/cm2 at the surface of each tank (Osram Biolux 72 fluorescent broad-spectre daylight tubes).

Fish mortality was recorded daily in all tanks, and dead fish was removed along with uneaten food and faeces. Unaccounted mortality (difference between observed mortality from collected fish and final numbers of surviving fish) was interpreted as cannibalism (also observed a couple of times).

Growth was determined in all tanks from wet weight (WW) at the end of the experiment, which lasted 37 days. Wet weight was measured on fish anesthetised with tricaine methanesulfonate (Finquel, MS 222). The cod juveniles were flipped on a sheet of towel paper and subsequently weighed using a Sartorius CP153 (Sartorius AG, Goettingen, Germany). Specific growth rates (SGR) were calculated from Ricker (1958) as percent daily increase in wet weight:

$$SGR = (e^{g} - 1) * 100\ \% \qquad \text{where} \qquad g = (\ln WW_{t} - \ln WW_{t_0})/(t - t_0)$$

From one tank of each treatment, water samples were collected once a week for determination of ammonia and bacteria (total and species). Hydrography (temperature, salinity, oxygen, pH, and redox) was also determined once a week with an YSI 556 MPS (YSI Inc., Yellow Springs, Ohio). To evaluate variation within triplicates, measurements were carried out in all three replicate tanks of the 10 times exchange rate of the UV-treatment.

Since pH of seawater is not expected to get below pH 7, the pH glass electrode was calibrated by 2-point calibration with IUPAC buffers at pH 7 and 10. The pH readings was therefore in accordance with the NIST scale (former NBS scale) which is most commonly used in marine aquaculture, although the Hansson's scale based on high ionic strength buffers may be more correct for measurement of pH in high ionic strength solutions like seawater (Hansson, 1973).

2.3. Ammonium analysis

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Water samples were analysed for total ammonium content (NH_4^+ and NH_3), using the salicylate-hypochlorite method (Bower and Holm-Hansen, 1980). Each sample was developed in triplicate before analysis. Absorbance of the ammonium reaction compound indophenol blue was detected by a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and total ammonium content determined from standard curves. Calculation of the unionized ammonia fraction (NH_3) was done from a minor correction of equation (8) of Fivelstad (1988) as compiled from Withfield (1974):

$$\% \, NH_3 = \frac{100}{1 + \text{antilog}\left(\left(9.245 + (0.002S)\right) + \left(0.0324(24.85 - t)\right) - \text{pH}\right)}$$

In this equation, S is salinity (‰) and t is temperature (°C). All references to mg/l for total or unionized ammonium are based on the full mole weight.

2.4. Flow cytometry

The water samples (18 ml) were fixed by adding 2 ml of a 40% formaldehyde solution. Total bacterial counts were determined using FacsCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with an air-cooled laser providing 15 mW at 488 nm and with standard filter set-up. The samples were diluted 5- to 10-fold in TE buffer and stained with SYBRGreen I (Molecular Probes Inc., Eugene, OR) for 15 min in the dark and at room temperature (Marie et al., 1999; Thyrhaug et al., 2003). The final concentration of SYBRGreen I in the samples was 2×10^{-4} of the commercial stock solution. The flow cytomer instrumentation and the remaining methodology followed the recommendations of Marie et al. (1999).

2.5. Polymerase chain reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE)

Water samples for PCR-DGGE analysis were taken once a week from the different water treatments and flow rates by filtering 18-20 ml water on 0.2 µm Dynagard hollow fibre syringe filters (Microgon InC. Laguna Hills, Ca). Total community DNA was further extracted from the syringe filters using a commercial kit for DNA isolation (Wizard®

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Genomic DNA Purification Kit, Promega, Madison, WI). Proteins were precipitated and DNA purified according to the manufacturer's protocol with modifications as described in Sandaa et al. (2003). The DNA pellet was dried and resuspended in 20 µl sterile distilled water. The extracted genomic DNA was used as target DNA in the PCR to amplify fragments suitable for DGGE analysis. The primer combinations EUBf (Giovannoni et al., 1990), with a GC-clamp, and PRU517r (Lane et al., 1985) were used with PCR conditions according to Sandaa et al. (2003). Clustering is based on the simple matching algorithm, while the dendogram was drawn using the complete link method.

DGGE was performed using a Dcode 16/16 cm gel system (BioRad, Herts, UK). PCR samples were loaded onto 8% (wt/vol) polyacrylamide gels in 0.5 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2 EDTA at pH 7.4). The linear gradient of urea and formamide ranged from 35 to 55 % denaturant. The electrophoresis was run at 60° C for 20 h at 60 V. The gels were stained for 1 h with a 1:10 000 dilution of SYBR Green II (Molecular Probes, OR) in distilled water before photography. DGGE bands to be sequenced were excised from the gel. The DGGE profiles from the last two sampling dates, 9 and 16 July, were analysed using the gel image analysis program Gel2K (Svein Norland, Dept. of Biology, University of Bergen). Clustering is based on the simple matching algorithm, while the dendogram was drawn using the complete link method.

The 500 bp amplicons were sequenced by cycle sequencing according to the protocol from Perkin Elmer using EUBf as a sequencing primer. Sequences were obtained on an ABI 377 sequence analyser (Perkin-Elmer Applied Biosystems, CA). The sequences have been deposited to GenBank with the accession numbers GU583742 through GU583788.

Analysis of 16S rDNA sequences was carried out by alignment to the closest relative in the nucleotide BLAST program (GenBank, NCBI) (Altschul et al., 1990). Additional 16S rDNA sequences from GenBank of closest relatives and type strains were used for the phylogenetic analysis**.** These sequences are; Alteromonas citrea (X82137), Alteromonas aurantia (X82135), Alteromonas sp. MOLA, (AM990812), Alteromonas sp. S11-B-8 (EU016171), Alteromonas sp. V4.BE.32 (AJ244758), Pseudoalteromonas sp. 'turbot' (AY227707), Pseudoalteromonas denitrificans (X82138) and Vibrio splendidus strain 03/012 (AJ874367), Listonella anguillarum (AM235737), Methylophilus methylotrophus (GQ411499), Saprospira sp. CNJ640 (AY527410), Roseobacter sp. UST050418-052 (FJ596360),

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Uncultured beta proteobacterium clone 9m05AISD07 (EF629704), Vibrio sp. V322 (DQ146983), and Vibrio sinaloensis strain CAIM 648 (EU043381). Phylogenetic relationships were inferred from multiple alignments by the use of CLUSTALX (Thompson et al., 1997), using the website www.phylogeny.fr (Dereeper et al., 2008). Alignment curation in the sequences was checked by using GBlocks (Castresana, 2000), while phylogeny was analysed using the PhyML 3.0 program. Supports for clades were estimated by using the approximate likelihood-ratio test with the settings SH-Like. The trees were drawn using the TreeDyn program (Chevenet et al., 2006).

2.6. Statistical analysis

Two-way ANOVA statistics were used to test for significant differences of water treatment and water exchange rate. Tukey HSD post hoc test was used to determine differences among all the variables. Mortality data, given as percentages, were arcsine transformed before statistical testing (Sokal and Rohlf, 1995). Differences among means were considered statistically significant at $P < 0.05$.

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3. Results

3.1. Survival and growth

Total mortality was between 8.5 and 60.5% among individual tanks. Total mortality (and hereby overall survival) was significantly modified by both water treatment ($P < 0.0001$) and water exchange rate ($P < 0.0001$). Observed mortality, averaged for the triplicates (Fig. 1A), was significantly affected by water flow rate ($P = 0.0001$), with the highest mortality occurring at the least water exchange. In contrast, cannibalism was lowest in the recirculation system (Fig. 1B), demonstrating the significant effect of water treatment on this mortality measure only ($P < 0.0001$). Within each tank, cannibalism accounted for a highly variable fraction of the total mortality, as cannibalism varied between 0.04 and 8.46 times that of the observed mortality. No interaction between water treatment and flow rate was evident for any of the mortality measures ($P > 0.5454$).

At experiment conclusion, average juvenile wet weight among individual tanks was between 0.73 and 1.67 g. Similarly, SGR was calculated to between 6.8 and 9.8% increase in weight per day among individual tanks. Average SGR among triplicate tanks was found to be significantly affected by both flow rate ($P < 0.001$) and water treatment ($P = 0.012$) as the juveniles from high flow rates and the UV-treatment showed elevated growth rates (Fig. 1C). No significant interaction between water treatment and flow rate was observed for SGR ($P = 0.1001$)

Both water treatment ($P < 0.0001$) and flow rate ($P < 0.0001$) had significant effects on the produced biomass density in the tanks (Fig. 1D), but interaction between water treatment and flow rate on biomass density was not significant ($P = 0.0519$). Among individual tanks, the biomass density at experiment conclusion varied between 1.8 and 7.5 g/l. Recalculating this to biomass-specific water exchange rates, it corresponded to a range of 2.9 to 22.6 l/g/day, with averages of triplicate treatments of 3.5-3.9 l/g/day (10X), 4.0-6.6 l/g/day (20X), 6.0-9.1 l/g/day (40X), and 10.5-16.7 l/g/day (70X).

Average oxygen saturation for the experimental period was significantly correlated with both survival (Fig. 2A, $R^2 = 0.48$, $P = 0.006$) and growth rate (Fig. 2B, $R^2 = 0.46$, $P = 0.0078$). High survival or SGR was associated with oxygen levels above 80% saturation and high

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water exchange rates. Similarly, average unionized ammonia (NH_3) concentration was negatively correlated with survival (Fig. 2C, $R^2 = 0.38$, $P = 0.019$) and SGR (Fig. 2D, $R^2 = 0.35$, $P = 0.026$). High SGR or survival was observed for NH_3 levels below 0.13 μM (0.0022 mg NH_3/l), occurring at high water exchange rates. No overall significant correlation was found between average pH levels during the experiment and survival or SGR (Fig. 2E, F). However, in the recirculation unit average pH throughout the experiment (7.38-7.61) was lower than in the flow-through treatments (7.71-8.02), and there was a strong tendency to improved survival or growth with increasing water exchange rate within each water treatment regime (Fig. 2E, F). This correlation was significant between average pH and survival for both flow-through treatments, and it was also significant for average pH and SGR in the untreated water. Finally, no correlation was found between survival and SGR (Fig. 2G), but high water exchange rates enhanced both growth and survival.

3.2. Bacterial counts and signatures by flow cytometry

Bacterial concentrations in the water, as counted by flow cytometry increased in all tanks and treatment from the beginning of the experiment to the end (Fig. 3A) The UV-treated and untreated water started at typical seawater concentrations (0.5-1 x10^6 mL^{-1}) and a further five to 10-fold increase was observed during the experimental period. The recirculated water tanks started with bacterial concentrations two to five times higher than the UV- and untreated tanks at the time of fish transfer, and ended up with 10 times higher concentrations in the end. Similarly, the mean side scatter (SSC) signal from the bacterial populations where stable in the untreated and UV-treated tanks, while the signal increased four to five times in the recirculated water tanks (Fig. 3B). However, this should not be directly linked to any physiological or genetic characteristics of the populations, but is rather an indication of a different development in the two systems.

3.3. Microbial community structure described by DGGE and sequencing

Cluster analysis of the DGGE profile from the different water treatments and flow rates at July 9th (Fig. 4A) separated the recirculation tanks, including the respective inlet water, from the flow-through systems at 0.55, with one exception. The exception, the highest flow rate of the untreated water (Un-70X), clustered together with the recirculation tanks. The number of bands in the 10X and 20X water exchange rates was higher compared to the 40X and 70X for

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all the water treatments (data not shown). At July 16th the cluster analysis of the DGGE profile from the different water treatments and flow rates (Fig. 4B) revealed two major groups at 0.64, one group consisting of the recirculation system, and the other group of the two flow-through systems with UV and untreated water. Similarly, band profiles were obtained from the inlet water of the recirculation system and the Re-70X tank. At 0.48 of the cluster analysis, the three water treatment systems composed three major groups with exception of tank UV-70X, which grouped together with the untreated tanks. The number of bands in the 10X and 20X water exchange rates was again higher compared to the 40X and 70X for all the water treatments (data not shown). It was not possible to obtain PCR products from the inlet waters of the flow-through systems, and these profiles were therefore not included in the cluster analysis.

Phylogenetic analysis of the DGGE sequences (Genbank accession numbers: GU583742-GU583788) showed that they fell into six distinct clusters (Fig. 5). From the top of the tree and downward, one sequence from the UV treatment (LB20) made up a cluster together with Saprospira sp. that belongs to the phylogenetic group Bacterioides. The next cluster consisted of six sequences (LB22, LS44, LB32, LB9, LB8, LB39) with low homology to any hitherto known bacterial strain. These sequences represent samples from all the three water treatments (Un, UV, Re). The unknown group clustered closely to the Gammaproteobacteria group, with a sequence difference of approximately 24%. However, 85 % of the DGGE sequences clustered within the Gammaproteobacteria divided into four distinct clusters denoted I to IV (Fig. 5). Cluster I (LB41, LB16, LB7, LB12, LB43) showed approximately 15% difference from the Vibrio group. Cluster I consisted of samples from all the three treatments. Cluster II consisted of 15 DGGE sequences together with bacteria belonging to the family Vibrioaceae. Most of these sequences (93 %) were from the DGGE profiles of samples from the Un and UV treatments. Cluster III made up five DGGE sequences from the Re treatment together with representatives belonging to the Alteromonas and Pseudomonas genera. Finally, cluster IV consisted of 15 homologous DGGE sequences, with approximately 26% sequence difference to the Alteromonas/Pseudomonas complex represented by cluster III. Moreover, 10 of these sequences (67 %) were DGGE profiles from the UV treatment.

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4. Discussion

The two mortality components, observed mortality and the unexplained mortality believed to be cannibalism, were affected differentially by water treatment and flow rate. The cause of death among observed dead fish was not explored. However, low flow brings on a number of changes in crucial water quality parameters related to oxygen supply, released metabolites, and microbial load. The metabolites will primarily include CO_2, which was not measured in the present study, and unionized ammonia (NH_3), which dissociate readily into the much less toxic ammonium (NH_4^+) in contact with water. NH_3 is a potent neurotoxin (Cooper and Plum, 1987) that is excreted into the water by ammoniotelic fish (Wilkie, 2002). The amount of NH_3 in the ammonium equilibrium is strongly dependent on pH and increases at higher pHs (Emerson et al., 1975; Whitfield, 1974). Moreover, a recirculation system with biofilter for nitrification of ammonium to nitrate will consume alkalinity with reduced pH as a result (Gundersen and Mountain, 1973). In addition, CO_2 from respiration will easily dissolve in water and add further to reduction of pH by producing carbonic acid. Acidification of the water in this way could have been avoided if the recirculation system has a very efficient CO_2 stripping component, but this was not the case in the present experiment.. Altogether, these factors accounted for the large drop in pH observed in the recirculation treatment, but which of the CO_2 and nitrification processes that was most important cannot be determined from the present data. But the recirculation system probably benefited from the low pH by reducing toxicity of unionized ammonium. In fact, despite that total ammonium was between 2.1 and 6.6 times higher in the recirculation tanks than in their respective flow rate tanks of the flow-through treatments, NH_3 was at the same level in all water treatments. There is a limited number of studies on acute toxicity of unionized ammonium in seawater, but levels between 0.09 and 3.35 mg/l NH_3-N (6.4-239.2 µM NH_3) have been suggested for marine fish in a review by Handy and Poxton (1993). Further, effects of chronic exposures may occur at 5-10% of the acute levels (Eddy, 2005). Without the pH effect in the recirculation treatment, NH_3 levels would have exceeded the levels of which effects from chronic exposure may be expected as calculated from the numbers above.

However, within each water treatment, there was a drop in pH of approximately 0.2 units from the highest to the lowest flow rate. This indicates an effect of CO_2 from respiration alone without nitrification. Increased CO_2 concentrations may reduce both survival and growth in fish (Ishimatsu et al., 2005), but since CO_2 was not measured in the present study,

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the exact outcome of CO_2 build-up cannot be assessed. Nevertheless, low water flow may have deteriorated water quality in general by increasing the levels of metabolites. This is further amplified as seen for NH_3, which was more abundant in the tanks with the lowest flow rate compared to the tanks with the highest flow rate (1.3 and 3.1-3.7 times more abundant in the recirculation and flow-through treatments, respectively). Although the observed NH_3 levels should not account for a particular increase in mortality or reduction in growth according to reported literature values for cod juveniles (Foss et al., 2004), the additive stress from accumulated metabolites and reduced oxygen levels may increase susceptibility to physiological perturbations which in turn may halt growth or lead to increased rates of infections from pathogens. Reduced oxygen may explain the effects on growth at the low flow rates as hypoxia in cod, particularly below 65% oxygen saturation, has been shown to decrease food consumption and explain most of the variation in growth, but with only a slight effect on food conversion efficiency (Cabot and Dutil, 1999). However, the levels of hypoxia in the present study were not low enough to affect survival in cod directly (Cabot et al., 2001). Thus, as both survival and growth was negatively affected by the low water exchange rate, resulting in reduced oxygen saturation, increased CO_2 concentration, and elevated NH_3 levels, this indicate that small but simultaneous changes in basic water quality parameters should not be overlooked regarding cumulative stress and fish welfare in ongrowing of marine juvenile fish. Still, very little is known about the mechanisms on how such stressors act, alone or in combination, chronically or acute, both at tissue, cell, or molecular levels.

More unexpectedly, motivation to eat conspecifics seemed to have been much less in the recirculation treatment compared to the other two flow-through treatments. The reasons for this are unclear, but several issues may be hypothesised for explaining the observed differences. Due to lower cannibalism in recirculation tanks, density of fish in these units became somewhat higher than in the tanks of the other two water treatments. There is also a tendency that cannibalism increases towards higher flow rate with more surviving fish. However, it is unlikely that density-dependent factors may explain the differences in the cannibalism, as survival numbers in the recirculation units were among the best at all flow rates compared to the other water treatments. Reasons for the reduced cannibalism may therefore be found elsewhere. First, recirculation of water also implies recirculation of metabolites and dissolved organic compounds, originating from the feed among others. Some of these water-soluble compounds may be amino acids that may interfere with the sensory

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system of fish. It may therefore be questioned if some kind of "smell" or "taste" did put the sensory system into satiation, and thereby reducing the motivation to eat conspecifics? Second, a considerable drop in pH was found in the recirculation treatment, and even a modest reduction in pH has shown to significantly alter the olfactory abilities of other marine fish (Munday et al., 2009). Suppression of taste nerve responsiveness has also been reported for intracellular pH changes resulting from metabolites (Yoshii and Yotsui, 1997). Whether young cod is susceptible to these effects is unknown, but it should be investigated if feeding behaviour and motivation change with pH. This may also have applications beyond aquaculture, as acidification of the ocean from increasing CO_2 loads is currently known to happen (Orr et al., 2005).

Clear differences were also seen in the bacterial community composition comparing the recycled water system (Re) and flow-through systems (UV, Un) (Fig. 4). The differences were reviled by generally cells with higher scatter and green fluorescence in the recycled systems, suggesting a larger cell-size compared to the cells detected in the flow-through systems (Fig. 4B). Accordingly, differences between water treatments were also observed in the DGGE patterns of 16S rDNA (Fig 4A and 4B). On a finer level of resolution (Fig. 5), the differences were detected within two clusters (II and III) both belonging to the Gammaproteobacteria class. Cluster III was only detected in the recirculation system, and showed highest affiliation to bacteria belonging to the Alteromonas/Pseudoalteromonas genera. In contrast, the flow-through systems were dominated of bacteria belonging to the family Vibrionaceae (Cluster II), with one exception; sequence LS18 that originated from the DGGE profile of the recirculation water system.

Dominance of Alteromonas/Pseudoalteromonas species associated with recirculation systems has previously been described (Fjellheim et al., 2007; Verner-Jeffreys et al., 2004). Species within Alteromonas/Pseudoalteromonas are known to be antagonistic (Hjelm et al., 2004; Holmstrøm and Kjelleberg, 1999; Riquelme et al., 1996). Including, several strains within these genera are known for their biofilm-forming capabilities, including solid surface attachment (Dang and Lovell, 2000; Egan et al., 2008; Pukall et al., 1999; Rao et al., 2005; Schäfer et al., 2000; Skovhus et al., 2004). The recirculation unit was initiated 16 weeks prior to the experiment to initiate biofilm formation. The formation of biofilms may provide a higher stability in the bacterial community in the tanks and also prevent pathogenic bacteria to colonise. The combination of surface attachment and antagonism are known for several

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bacterial strains isolated from tank walls (Bruhn et al., 2006; Hjelm et al., 2004; Porsby et al., 2008). In this context, Hjelm et al. (2004) points to the differential biofilm-formation of pathogen-antagonising bacteria in larval tanks as a possible explanation for the large inter-tank variability in survival within marine hatcheries. In the present study, the coefficient of variation (Sokal and Rohlf, 1995) of survival among the triplicate tanks of each water exchange rate was lower in the recirculation treatment compared to the two flow-through treatments, particularly if flow rates from 20X and larger are considered: 1.7-7.3% in Re, 8.5-16.7% in UV, and 5.4-26.9% in Un. Thus, we might speculate if a possible successful biofilm formation in the recirculation system created a more stable microbial environment and hence lower variation in survival among the triplicates.

The detection of Pseudoalteromonas/Alteromonas in the recirculation system may also be explained by their extreme long-term starvation capacity that enable them to adapt to nutrient depletion in a state termed as starvation-survival (Cappello et al., 2008; Givskov et al., 1994; Nissen, 1987; Pernthaler et al., 2001). During the period of conditioning only ammonium and no carbon-based substrate were added to the recirculation unit. Thus, Pseudoalteromonas/Alteromonas may have adapted to a starvation-survival strategy in the recirculation water system during this period. High concentration of ribosomes during extended periods of non-growth makes members of the Pseudoalteromonas/Alteromonas genera able to rapidly respond to nutrient addition (Allers et al., 2007; Eilers et al., 2000a; Pernthaler et al., 2001). The subsequent addition of fish, feed and faeces represented a huge organic load to the rearing tanks. When nutrients in the form of carbon are in excess an increase in cell size has been observed within the Alteromonas genus (Allers et al., 2007). In the present study cells with large cell-size were detected in the recirculation system only and may be attributed to the detection of Alteromonas. Another major difference between the two water systems was bacterial abundance, observed as 2-5 times higher in the recycled water tanks compared to the flow-through system in the start of the experiment, and 10 times higher in the end of the experiment. Apart from the head start of this recirculation system and the circulation of bacterial biomass, a higher ammonium concentration was also detected in the recirculation system. High ammonium concentrations are particularly favourable for growth of colony-forming Gammaproteobacteria (Eilers et al., 2001) and may further have contributed to the higher bacterial abundance observed in this system.

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In the flow-through systems bacteria belonging to the family Vibrionaceae were detected. As with the the Pseudoalteromonas/Alteromonas genera, the Vibrio genus is rarely detected in situ (Eilers et al., 2000b). However, they are easily cultivated in response to nutrient addition, as demonstrated in seawater enclosures (Allers et al., 2007; Eilers et al., 2000a; Lebaron et al., 1999; Øvreås et al., 2003). Species within the Vibrionaceae family are frequently associated with disease in farmed fish (Thompson et al., 2004; Toranzo et al., 2005). Vibrio sp. was found as the dominating genus associated with moribund cod larvae by Brunvold et al. (2007). Although, the majority of sequences in the present study could not be attributed to recognised species of the genus Vibrio, some of the sequences associated with the flow-through systems showed 99% homology to Vibrio splendidus and Vibrio sinaloensis. Recently, results have shown that V. splendidus is able to cause mortality in cod larvae (Reid et al., 2009). V. sinaloensis was originally isolated from the spotted rose snapper Lutjanus guttatus (Gomez-Gil et al., 2008), however it was associated with mortality. In addition, the phylogenetic analysis displayed a group with high sequence similarities to Vibrio anguillarum (Fig. 5). V. anguillarum is a well described pathogen of cod larvae, juveniles and adults (Reid et al., 2009; Samuelsen et al., 2006; Sandlund and Bergh, 2008; Toranzo et al., 2005). In the present study, no clear correlation was observed between the presence of fish pathogenic bacteria belonging to species within the Vibrionaceae family and increased mortality. However, cannibalism was significantly higher in the flow-through systems, and it may be speculated if Vibrio infections might have weakened the larvae, which in turn have become more prone to be eaten by conspecifics. Studies have shown that differences in health status may affect the frequency of cannibalism amongst reared cod juveniles (Folkvord, 1991). Nevertheless, it must be emphasised that caution should be exercised in drawing conclusions about whether these bacteria are involved in disease and mortality as the analysis were based on a short region of the 16S rRNA gene. Studies have reported the limitations of 16S rDNA-based analysis, due to low variation in the 16S rRNA gene that makes it difficult to discriminate between bacterial species (Case et al., 2007). Polymorphism between repeated 16S rRNA genes within genomes of Vibrio are known to exist (Moreno et al., 2002), and the use of DGGE may further add to this problem in obtaining reliable sequences for phylogenetic analysis (Sekiguchi et al., 2001, Brunvold et al., 2007).

Dominance of bacteria belonging to the Gammaproteobacteria class is frequently associated with intensive rearing of marine larvae, and correlation with lower larval growth and survival is reported (Bjornsdottir et al., 2009; Brunvold et al., 2007; Nakase et al., 2007; Schulze et

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al., 2006). This correlates to the findings in our study where the different water treatment systems had a dominance of bacteria belonging to the Gammaproteobacteria class. The bacterial taxa detected in our study are commonly characterised as opportunistic r-strategists. Thus, empirical evidence from other studies, as well as the present work, indicates a selection from a more typically K-selective community normally dominating in seawater (Schut et al., 1997), towards bacteria with a more r-selective strategy in marine larval tanks (Vadstein et al., 2004). In general, it appears important to apply water treatments systems to avoid the tendency towards dominance of r-strategic Gammaproteobacteria. In this sense, water treatment may be beneficial as part of a strategy to obtain microbial control. However, the use of a recirculation system imposes a challenge to the establishment of a desirable bacterial community structure in the unit, as unfavourable bacterial communities may also be established and circulated in the system. Furthermore, the community structure is altered by the addition of fish, feed and faeces, favouring r-selected strains. In this context the effort should be directed towards the establishment of a robust system also including non-pathogenic r-selected strains, able to withstand such organic pulses. Enhanced biofilm formation, possibly by the action of probiotic bacteria with biofilm-forming preferences to solid surfaces could thus be a possible strategy.

No significant effects of flow rate on bacterial abundance and community composition were found. However, a slightly higher number of bands were observed in the DGGE profiles associated with the lower flow rates (10X, 20X) compared to the higher flow rates (40X, 70X), which may indicate a higher bacterial diversity in the lower flow rates. It is important to keep in mind that conclusions about diversity based on DGGE should only be viewed as an indication rather than an absolute measurement (Marzorati et al., 2008). The results from flow cytometry analysis also revealed a higher bacterial abundance in the low flow rates compared to the high flow rates (data not shown). On average, the bacterial number was approximately twice as high in the lowest (10X) compared to the highest (70X) flow rate (data not shown). This trend corresponds with Opstad et al. (1998) who observed a higher number of bacteria in the tanks with lowest flow rate when studying bacterial abundance in relation to flow rate in halibut larval rearing tanks. Increasing flow rate will have a dilution effect on organic substrates and bacterial biomass, and the observed tendencies may therefore be expected. However, it is unclear if the flow-related divergences in microbial densities and communities may have affected larval survival and growth. Nevertheless, based on the actual survival and growth data, flow rate should be at least 40 tank volumes per day. Recalculating

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this to biomass-specific water exchange rate in the present experiment, this corresponds to 6.0, 8.8, and 9.1 l/g WW/day for the recirculation, UV-treated, and untreated systems, respectively. Thus, an average biomass-specific flow rate of 8.0 l/g WW/day may be suggested as a guideline for flow requirements during early juvenile stages of Atlantic cod.

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Figure 1. Observed mortality, unexplained mortality (cannibalism), specific growth rate (SGR), and biomass density among the various water quality and flow rate treatments at end of the experiment. Bars are averages of three replicate tanks with error bars as standard deviations. Similar lowercase letters above the bars denote group homogeneity determined from the Tukey HSD post-hoc test.

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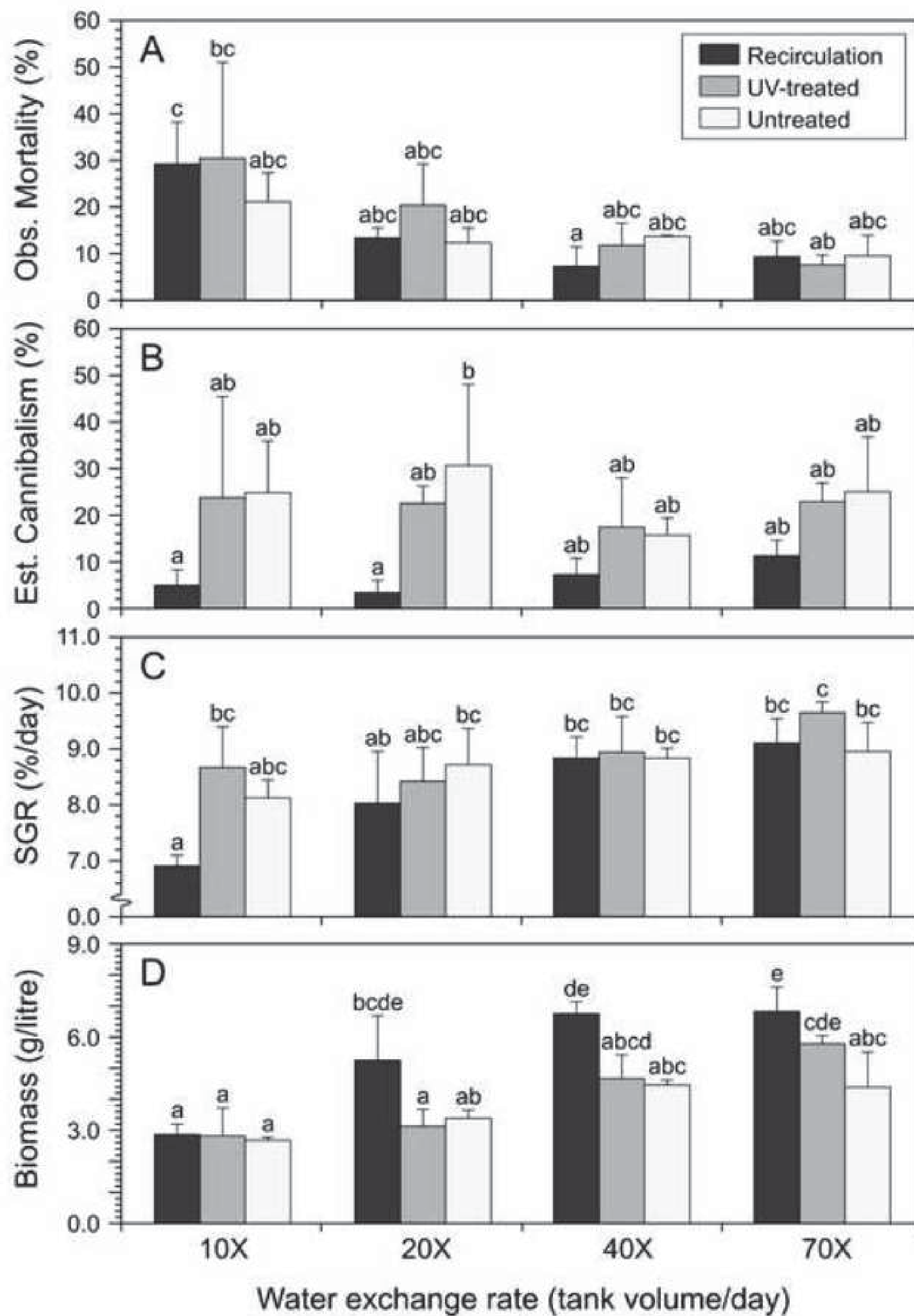

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Figure 2. Relationships between survival, growth, oxygen saturation, unionized ammonia, and pH. Data points are averages over the whole experimental period. The water exchange rates (10X, 20X, 40X, and 70X of the tank volume/day) are indicated for the three water treatment regimes.

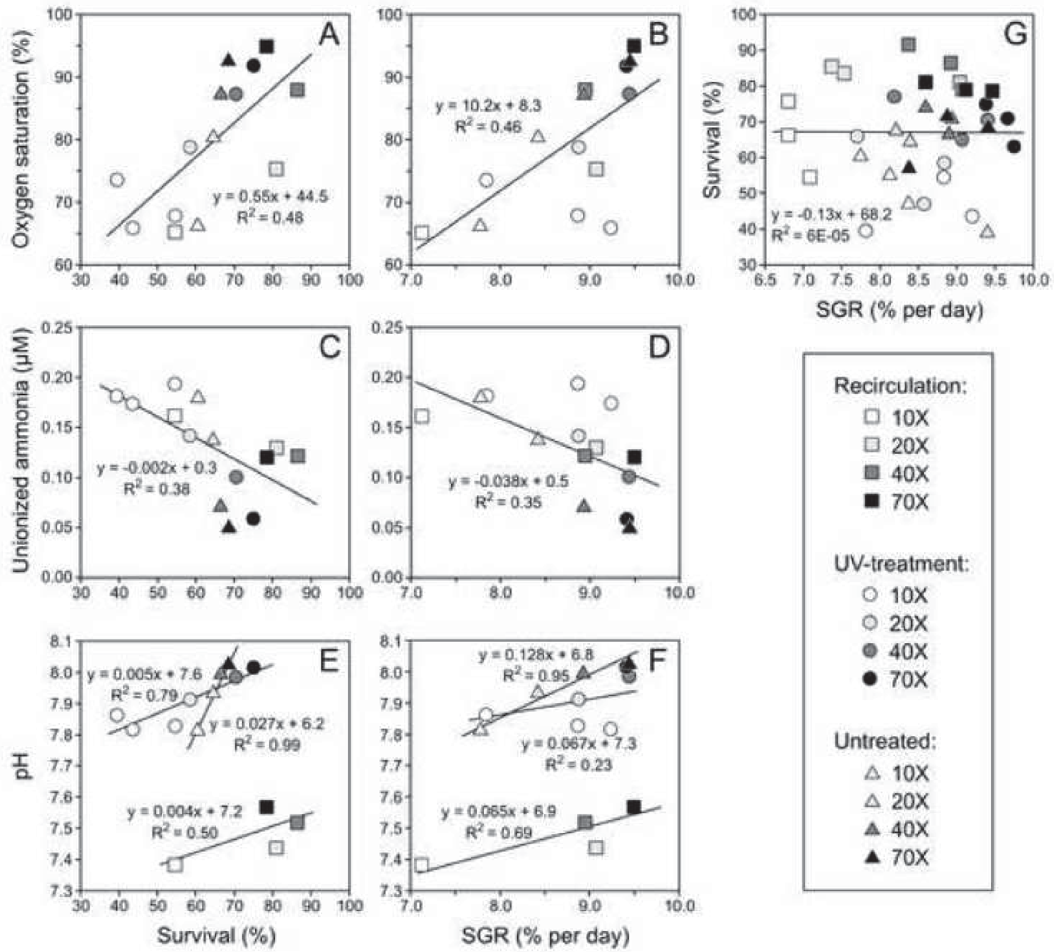

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Figure 3. Flow cytometer data of bacteria in the experiment. Data points are average of the four flow rates within each water treatment, and error bars are standard deviations. A) Total number of bacteria, B) Mean SSC signal per bacterial cell.

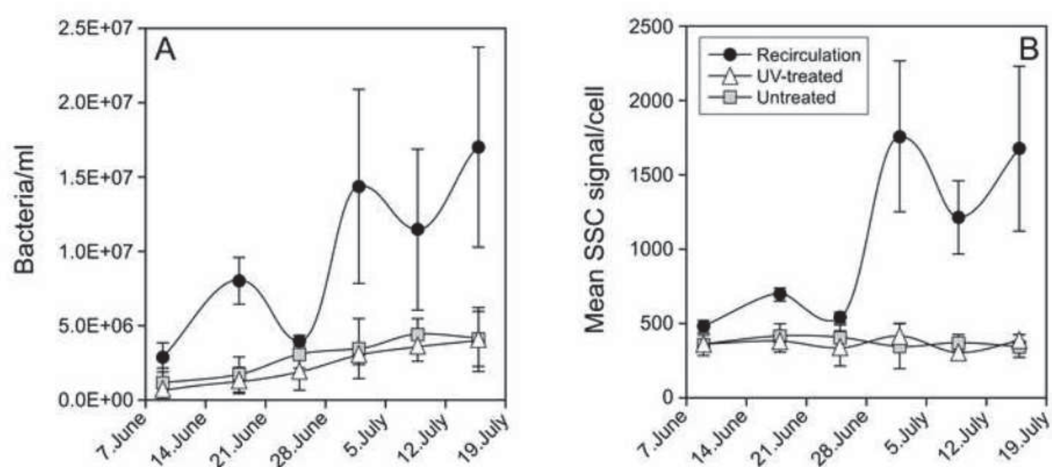

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Figure 4. Cluster analysis of the DGGE profiles analyzed from the experiment at 9th of July (A) and 16th of July (D). Water treatments (Recirculation: Re, UV treatment: UV, Untreated: Un) are shown together with flow rate (10X, 20X, 40X, 70X). Inflowing water are included for the Re treatment, only. FCM dot plots from a typical Re tank (B) and a tank representing the typical UV and Un situation (C) show green fluorescence vs. SSC signal from single bacterial cells (red dots) and other non-bacterial particles (black dots). The two plots visualise signals characteristic of different bacterial populations.

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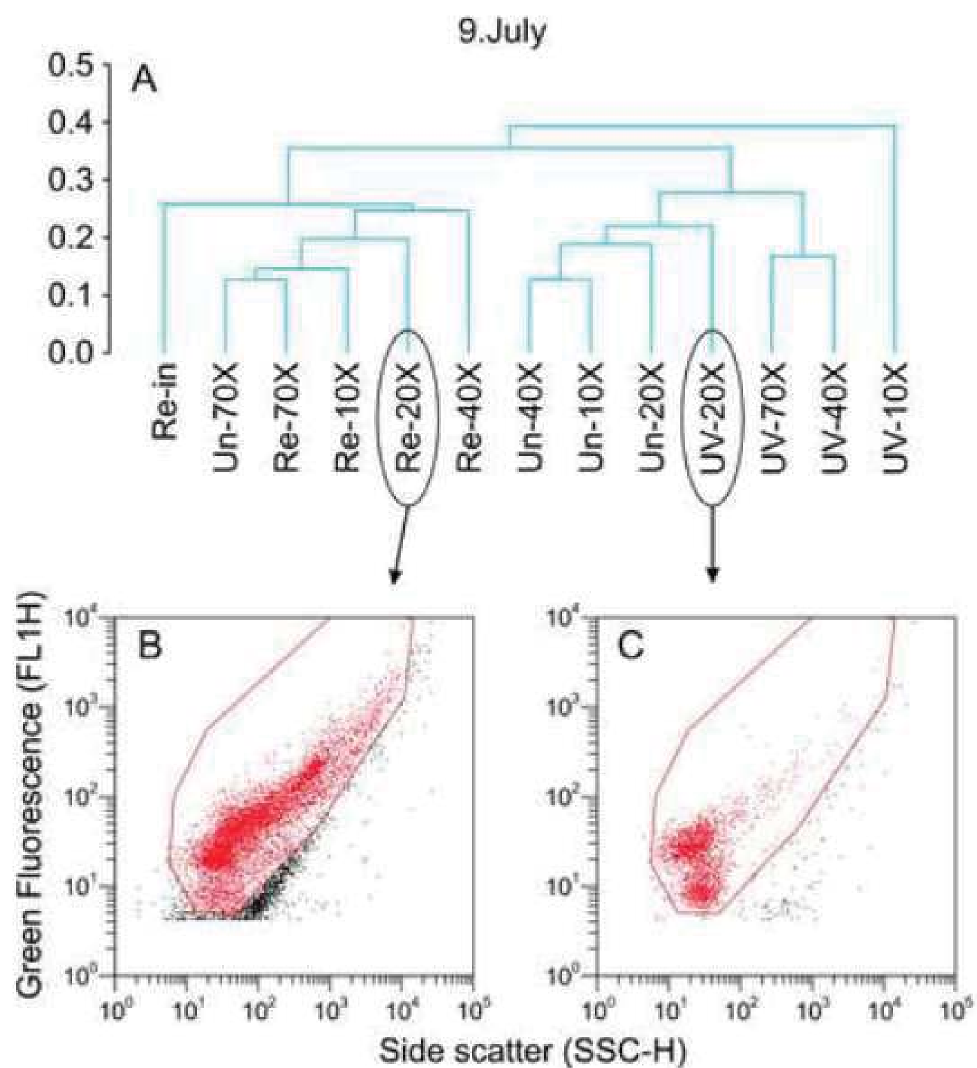
9.July
A
0.5
0.4
0.3
0.2
0.1
0.0
Re-in
Un-70X
Re-70X
Re-10X
Re-20X
Re-40X
Un-40X
Un-10X
Un-20X
UV-20X
UV-70X
UV-40X
UV-10X
B
C
Green Fluorescence (FL1H)
Side scatter (SSC-H)

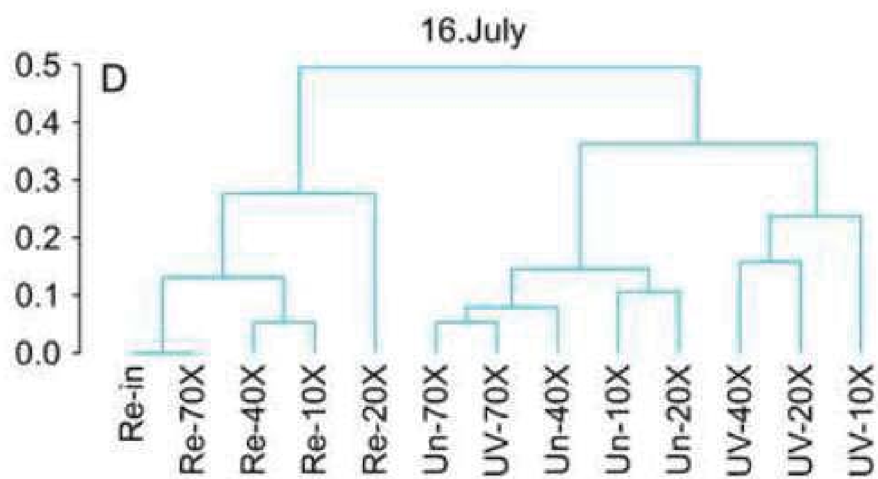
16.July
D
0.5
0.4
0.3
0.2
0.1
0.0
Re-in
Re-70X
Re-40X
Re-10X
Re-20X
Un-70X
UV-70X
Un-40X
Un-10X
Un-20X
UV-40X
UV-20X
UV-10X

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Fig. 5. Phylogenetic affiliation of 16S rDNA sequences derived from DGGE profiles associated with the recirculated (red), UV treated (blue), and untreated (green) water from samples collected from the whole sampling period. Fifteen marine bacteria (black) recovered from GenBank are included as reference sequences and given in section 2.5. Supports for clades were estimated by using the approximate likelihood-ratio test with the settings SH-Like. Scale bar represents 0.2 substitutions per site.

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1.0 LB20
Saprospira sp. CNJ640
Roseobacter sp. UST0504
1.0 Methylophilus methylotrophus
Uncultered beta-proteobacterium
1.0
0.9 LB22
LS44
LB32
0.8 LB9
LB8
0.8 LB39
0.7 LB41
LB16
LB7
1.0 LB12
LB43
I
1.0 LS66
Vibrio splendidus
Listonella anguillarum *
1.0 LB23
LS26
LS60
LS31
LS10
LS52
LS18
0.9
1.0
0.8 Vibrio sp. V322
LS39
LS23
LS15
Vibrio sinaloensis
LS34
LS68
LS55
II
LB4
LB13
1.0 Alteromonas sp. S-11-B-8
Alteromonas sp. MOLA
0.9 Alteromonas sp. V4.BE.32
1.0
0.9
0.8
LB29
0.8 LB14
Pseudoalteromonas sp. turbo
Pseudoalteromonas denitrificans
0.9
0.9
0.7
Alteromonas citrea
0.7 Alteromonas aurantia
LS53
LB1
III
0.8
LS35
LS54
0.8
LS65
LS64
LS37
1.0 LS22
LS51
LS56
LS17
LS33
LS41
LS57
LS25
LS59
LS4
IV
0.2

Bacteroides
Alpha-proteobacteria
Beta-proteobacteria
Unknown bacterial group
Gamma-proteobacteria