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Water treatment as an approach to increase microbial control in the culture of cold water marine larvae

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List of papers

Paper 1
Attramadal, K.J.K., Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2011. Recirculation as a possible microbial control strategy in the production of marine larvae. *Manuscript submitted*.

Paper 2
Attramadal, K.J.K., Øie, G., Størseth, T.R., Alver, M.O., Vadstein, O., Olsen, Y., 2011. The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. *Manuscript submitted.*

Paper 3
Attramadal, K.J.K., Tøndel, B., Salvesen, I., Øie, G., Vadstein, O., Olsen, Y., 2011. Ceramic clay reduces the load of organic matter and bacteria in marine fish larval culture tanks. *Manuscript submitted.*

Paper 4
Hess-Erga, O-K., Attramadal, K.J.K., Vadstein, O., 2008. Biotic and abiotic particles protect marine heterotrophic bacteria during UV and ozone disinfection. Aquatic Biology 4, 147-154.

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Paper 1 to 4

1 Introduction

This thesis is part of the strategic research programme CODTECH - A process oriented approach to intensive production of marine juveniles with main emphasis on cod. The programme aimed to build a basis for the next generation of technology for production of marine juveniles by incorporating methods of control and automation from other process industries. Pursuing this goal required insight and competence on the development and biological demands of marine larvae and on the process of production. The research involved the quality and development of larvae, their nutritional needs, microbial interactions and physiochemical factors in the rearing process. Atlantic cod (*Gadus morhua* L.) was used as a model species, but the results are applicable to other marine species that is first fed with live plankton.

The scope of this thesis was to develop methods of water treatment to increase control and improve the general microbial environment in the rearing water of cod larvae in intensive production. Other parts of the CODTECH research programme are presented in the NTNU theses: "Selection and administration of probiotic bacteria to marine fish larvae" (Anders Jón Fjellheim 2006), "Modelling, instrumentation and control in marine larviculture" (Morten Omholt Alver, 2007), "Functional development and digestive responses in cod (*Gadus morhua*) larvae to nutritional variations in formulated feeds" (Per-Arvid Wold, 2007), "*Calanus finmarchicus* as a potential basic feed ingredient" (Geir Solgaard, 2008) and "Gene expression in cod larvae as a response to larvae-bacteria interactions" (Torunn Forberg, in prep.).

1.1 Pelagic marine fish larvae

Marine species with pelagic larvae release a great number of offspring supplied with relatively small yolk reserves. Compared to other successfully farmed species like salmon, the pelagic marine larvae are hatched and forced to start feeding themselves at a much earlier developmental stage. The life history strategy has some important consequences for the cultivation of marine juveniles. The pelagic larval stage is characterised by an intense development of organs, gut and immune system, which makes the larvae especially vulnerable to unfavourable nutritional, physiochemical and microbial conditions. The current production of species like cod, sea bass and turbot are based on an initial period with live feed like rotifers, *Artemia* or copepods. The lack of a functional stomach during the pelagic larval stage is an important reason for the difficulties of developing a practically and economically viable formulated feed product to fully replace live prey organisms as first feed (Kjørsvik *et al*., 2004). Newly hatched pelagic marine fish larvae do not have a functional specific immune system. They rely on their general immune defence to combat infections, and are sensitive to microbial interference (Vadstein, 1997, Schrøder *et al*. 1998). Eggs are commonly surface disinfected (Salvesen and Vadstein, 1995), and the larvae depend on the microbial community in the surrounding water and feed for the primary colonisation of skin and gut.

In aquaculture the early larval stage is a costly period due to the dependence on labour-intensive production of live feed. The young larvae are sensitive to stress, infections and fluctuations in the physiochemical environment, but have a high growth potential. Larvae are adaptable to suboptimal conditions within certain limits for each variable, but compensation requires energy that could otherwise have been used for growth. If compensation for suboptimal conditions requires too many resources, the larva cannot cope and the investment is lost. In addition, the quality of the fish during early life stages will probably be reflected in later stages of production. Efforts to improve quality and performance during the early stages

of juvenile production are therefore worth while. Rosenlund and Halldorsson (2007) have published an overview of the research and commercial developments in cod juvenile production.

1.2 Water treatment in the production of marine cold water juveniles

Marine hatcheries represent high value, low waste and complex biological systems. Inflowing water is screened to remove particles, heated or cooled, aerated and often disinfected to prevent intrusion of pathogens. Live prey is fed in high concentration several times a day. The water exchange rate of the rearing tanks is commonly kept low to retain the valuable live feed and to protect larvae from mechanical stress. Low water exchange rates in the rearing tanks lead to accumulation of particles, organic matter, bacteria and metabolic waste products and at the same time limits the effect of external water treatment. This is a reason why it may be an advantage to be able to treat the water directly in the fish tanks during the first weeks after hatching (Paper 3). The biomass, oxygen consumption and waste production of the rearing tanks are high compared to the ocean and low compared to ongrowing systems. In hatcheries the load of waste is increasing fast as the fish grow and feed in an exponential manner.

1.2.1 "Green water" and addition of particles

Most marine hatcheries condition the rearing water with microalgae, be it live algae cultures or commercial concentrates (algae paste), during the first period when the larvae are fed rotifers. This so called "green water" technique has been shown to have a beneficial influence on survival and growth of marine fish larvae (Howell, 1979, Naas *et al.*, 1992, Reitan *et al.*, 1993, Salvesen *et al.*, 1999, Lazo *et al.*, 2000). The positive effects of "green water" were attributed by Naas *et al.* (1992) to turbidity improving foraging conditions by affecting prey contrast and larval distribution. Microalgae may also have a beneficial nutritional impact as feed for live prey and larvae (Reitan *et al.*, 1993, 1994, 1997), and may stimulate digestive enzyme activity (Cahu *et al.*, 1998). Moreover, addition of live microalgae has been shown to have a positive effect on the microbial environment of the culture water (Skjermo and Vadstein 1993, Salvesen *et al.*, 1999) and may accelerate the initial bacterial colonisation of the fish gut (Bergh *et al.*, 1994). Recently, the addition of inorganic clay is increasingly used to condition the water in first feeding of halibut (Harboe and Reitan, 2005, Björnsdóttir, 2010) and wrasse. Clay provide turbidity without contributing organic matter and is more cost efficient than the application of both algae and algae paste.

1.2.2 Types of water treatment system

There are two different ways to influence the quality of the inflowing water: treatment of the intake water in flow-through systems (FTS) or continuous restoration and reuse of the culture water in recirculating aquaculture systems (RAS).

The intake water in a FTS is treated to adjust the quality of the water to the requirements of the cultured species and stage and to protect the larvae from pathogenic organisms. Common steps of treatment of intake water in a FTS are particle removal, temperature regulation, aeration/degassing and disinfection. The quality of the water that is pumped into intensive aquaculture systems is important, but what happens with the water inside the farm is sometimes even more decisive for the water quality experienced by the cultured organism. Most forms of water treatment affect other factors than the one that is targeted. For example, heating increases temperature, but it also affects the equilibrium of gasses in the water.

In RAS the water is retrieved from the culture tanks and treated to remove waste products and may be supplied with oxygen to replace what is lost to metabolism. Water treatment in RAS

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is typically targeted at reduction of particles, dissolved organic matter (DOM), bacteria, ammonia, nitrite and carbon dioxide which is produced in the rearing tanks when larvae are fed and respire. In this thesis, the term RAS is used for systems including a biofilter and with a relatively high degree of reuse of water (<10 % new water added daily).

RAS and FTS offer different advantages. Which system type that works out best depends on specific requirements of the species and stage in question and the characteristics of the location. The small volumes of water going into and out of RAS compared to FTS makes it easier to treat and control. RAS are to a higher degree independent of a good quality water source, and offer higher location flexibility. Conservation of temperature means less energy may be spent on heating the water in RAS compared to in FTS. The opportunity to maintain a stable high temperature through the winter may be of economical importance in cold water marine hatcheries, as it allows for optimal growth throughout the year. Initial costs of investment are normally higher for RAS than for FTS and in most cases RAS involve mechanically sophisticated and more complex components and require a higher level of competence than FTS.

1.2.3 Particles

Hatchery waters typically have a quantitative predominance of small organic particles (Rueter and Johnson, 1995) which tend to accumulate in RAS (Chen *et al.,* 1993a, McMillan *et al.*, 2003, Patterson and Watts, 2003a,b). The particles originate from microalgae, the defecation process and dead and decaying feed organisms and larvae. The effects of turbidity in fish culture water depend on the nature and concentration of the suspended particles. Addition of several species of microalgae, concentrated microalgae paste or ceramic clay to fish tanks during early start feeding has been shown to have beneficial effects on the performance of marine fish larvae (Howell, 1979, Naas *et al.*, 1992, Reitan *et al.*, 1993, 1997, Salvesen *et al.*, 1999, Paper 3). For some anadromous and fresh water fish high concentrations of suspended particles have been shown to cause stress and reduce disease resistance (Redding *et al.*, 1987, Bullock *et al.*, 1994, Lake and Hinch, 1999). Organic particles are a source of substrate for bacteria and contribute to oxygen consumption and waste production when degraded. Particles in the water decrease the disinfection efficiency of UV and ozone (*1.2.7 Disinfection*, Paper 4). In sea water, protein skimming (foam fractionation) is an efficient and gentle way to remove small suspended solids and organic molecules (Lawson and Wheaton, 1980, Weeks *et al*., 1992, Chen *et al*., 1993a,b, Chen *et al*., 1994a,b). In a protein skimmer the water is brought into contact with fine bubbles. Substances gathering in the air-water interface are carried to the top of the skimmer where the foam is removed.

1.2.4 Gasses

Oxygen is consumed by respiration of fish, live feed and micro-organisms breaking down organic material and by nitrifying bacteria (Watson, 1971). Oxygen diffusion through fish gills depend on the difference in oxygen partial pressure between water and blood (Randall and Daxboeck, 1984). At the same partial pressure in the air in contact with the water, oxygen concentration decreases with increasing water temperature. In addition, oxygen consumption increases as metabolism increases with temperature. The oxygen consumption may be higher in fed compared to unfed fish (Sigholt *et al*., 1993) and stress increases the oxygen demand. In general, a minimum constant level of 5 mg O_2 L^{-1} is satisfactory for most stages and activities of fish provided that other environmental factors are favourable (Poxton and Allouse, 1982). It should be noted that low oxygen concentration may increase the toxicity of several harmful compounds. Aeration and oxygenation can be used to restore oxygen concentration in the water.

Carbon dioxide is a product of the respiration of fish and heterotrophic bacteria decomposing organic matter and it affects pH through the bicarbonate system. In sea water with a pH ~ 8, most of the carbonate exists as bicarbonate (HCO_3^-). At lower pH, relatively more of the carbonate exists as CO_2. Excess CO_2 may be removed by aeration and degassing of the water (Summerfelt *et al.*, 2003). Problems with too high CO_2 concentrations in tanks may arise in RAS, and in systems with oxygenation. Sublethal effects may occur from 10-20 mg CO_2 L^{-1} (Grøttum and Sigholt, 1996). At high concentrations of CO_2 in the water, fish have problems with gill CO_2 excretion, which leads to an elevated blood CO_2 concentration and a drop in blood pH. Lowered pH reduces the oxygen affinity of the hemoglobin (Bohr effect) and an elevated CO_2 concentration in the blood reduces oxygen binding capacity of the hemoglobin (Root effect). Intracellular acidosis affects a number of physiological processes to the extent that it may be lethal. In addition, CO_2 itself may be toxic to animal cells (Max, 1991). Ishimatsu *et al.* (2004) attributed higher CO_2 tolerance of larvae compared to juveniles to the development of gill lamellae and the resulting dramatic increase in the surface area available for diffusion, in contrast to the diffusion-limited gas transfer across the body surface in the early developmental stage.

Supersaturation of nitrogen gas may be hazardous to marine fish larvae (King and Nardi, 2002, Gunnarsli *et al.*, 2009). Fish that stays in total gas supersaturated water will have supersaturated blood. Nitrogen is biologically inert and when the pressure is reduced because of friction in the blood vessels, degassing and harmful bubble formation occurs in the circulatory system and body cavities. Gas supersaturation can arise in water that has been heated, pumped from a depth without aeration or where air or oxygen is added under pressure. Supersaturated water may be degassed in trickling filters or in vacuum aerators where excess gas is constantly being removed by low pressure.

1.2.5 pH

A safe pH-range for fish is 5-9, while the optimal lie between 6.5 and 8.5 (Brownell, 1980a, Poxton and Allouse, 1982). Nitrifying bacteria in biofilters are pH sensitive, and die below pH 6 (Lucchetti and Gray, 1988). A pH below 7 reduces the activity of nitrifying bacteria, while the optimal range is 7.5-8 (Watson, 1971). In general, in natural sea water, pH is relatively stable and in the range of 7.5-8.4, often about 8.1. In fish culture water pH is lowered by CO_2 produced from the respiration of fish and bacteria. Metabolism, and CO_2 production, increases as the fish grow in the system. In the RAS, pH and alkalinity also become lowered by nitric acid formation by nitrification of ammonia which releases protons in the reaction. A high pH increases the fraction of unionized ammonia, whereas low pH increases carbonate toxicity because of a relatively higher CO_2 fraction. The pH can be restored by addition of for example calcium carbonate, sodium bicarbonate or sodium hydroxide.

1.2.6 Nitrogenous waste products

Protein degradation in fish produces nitrogenous waste products that are released to the rearing water through gill diffusion, cation exchange over gills and in urine and excrements. In addition, microbial degradation of organic matter contributes to the nitrogenous load (Ellner *et al.*, 1996). In FTS the waste products are diluted, whereas RAS depend on bacteria mediated conversion of ammonia to nitrite and further to the less harmful nitrate in the biofilter. A biofilter is a reactor with a large surface area for biofilm formation. Ozone may also oxidate nitrite to nitrate (Rosenthal and Otte, 1979, Paller and Lewis, 1988, Borges *et al.*, 2003). Both un-ionised ammonia (NH_3) and nitrite (NO_2) are toxic to marine fish larvae and juveniles at low concentrations, with sublethal effects like reduced growth rate (Brownell,

1980b, Handy and Poxton, 1993, Parra and Yufera, 1999, Foss *et al.*, 2004, Siikavuopio and Sæther, 2006, Björnsson and Olafsdóttir, 2006). Cod juveniles showed reduced growth rates at concentrations above 0.06 mg NH_3-N L^{-1} (Foss *et al.*, 2004) and at 1.0 mg NO_2-N L^{-1} (Siikavuopio and Sæther, 2006).

A stable nitrifying biofilm is harder to maintain in systems with cold (Wortman and Wheaton, 1991, Zhu and Chen, 2002) and saline water (Nijhof and Bovendeur, 1990, Chen *et al.*, 2003). Especially the establishment and response of the nitrite to nitrate conversion step may be slower in marine biofilters (Manthe and Malone, 1987). In addition, larval rearing systems are operating with low, but exponentially increasing levels of wastes as the fish grow, which further complicates the operation (Zhu and Chen, 1999, Gutierrez-Wing and Malone, 2006).

1.2.7 Disinfection

Strong barriers against pathogens from external sources are necessary to maintain biosecurity in intensive aquaculture. Two of the most common treatment technologies for water disinfection are UV irradiation and ozonation.

Ozonation

Ozone (O_3) is a powerful oxidising agent which may be used to inactivate bacteria and viruses, including fish pathogens, in freshwater and sea water (Wedemeyer and Nelson, 1977, Blogoslawski *et al.*, 1978, 1992, Sugita *et al.*, 1992, Liltved *et al.*, 1995, Arimoto *et al.*, 1996, Grotmol and Totland, 2000). Ozonation of sea water quickly results in a series of red-ox reactions with the production of several reactive intermediates. The main ozone demand in sea water is due to its reaction with bromide to form bromine compounds. Bromide ions (Br^-) are oxidised to hypobromite ions (OBr^-) that hydrolyses to hypobromous acid (HOBr). The sum of HOBr and OBr^- is the biocidal bromine. At a pH of 8, which is common for sea water, hypobromous acid predominates as the most important disinfectant with a half-life of hours to days dependent on light conditions and water quality (Liltved *et al.*, 2006). Prolonged ozonation can further oxidise OBr^- to form bromate ions (BrO_3^-), which is persistent and toxic. The residual oxidants produced by ozone in sea water can be highly toxic to fish and live feed organisms (Ozawa *et al.*, 1991, Allen Davis and Arnold, 1997, Grotmol and Totland, 2000). The amount of residual oxidants can be reduced by sodium thiosulphate (Hemdal, 1992), removed by active carbon filters (Ozawa, 1991, Kobayashi *et al.*, 1993), addition of low levels of hydrogen peroxide or exposure to high intensity UV light (Summerfelt, 2003).

The measurement of residual ozone in sea water is complicated due to the rapid and continuous formation of oxidant products. The level of dissolved ozone can be estimated indirectly by measuring the oxidation reduction potential (ORP) or by the colorimetric N,N-diethyl-p-phenylenediamine (DPD) method. Measurements of ORP may be obtained continuously with electrodes, which is useful for controlling ozone addition to culture water. However, the ORP is not a direct reflection of the ability of the treated water to disinfect (Tango and Gagnon, 2003). The DPD "total chlorine test", which gives the concentration of total residual oxidants (TRO) in units of Cl_2 L^{-1}, is recommended to obtain the disinfection dose (Buchan *et al.*, 2005). The disinfection dose (C × t = concentration × time) is commonly calculated from the average TRO concentration during the contact time.

Water quality affects the efficiency of ozonation. TRO is consumed when ozone and bromine react with a variety of constituents in the sea water, including organisms. Because the concentration of TRO that are required to obtain a certain degree of inactivation depends on

the content of inorganic and organic matter in the water (Paper 4), there are no universally accepted standards for the amount of ozone necessary to add to achieve a certain effect.

The motivation for adding ozone to RAS is normally not disinfection as the organic load and nitrite in RAS process water demand large doses of ozone (Summerfelt, 2003, Tango and Gagnon, 2003). The dissolved ozone reacts so fast that a residual cannot easily be maintained to provide sufficient ozone concentration and contact time for disinfection. In addition, high levels of ozonation are often avoided in sea water RAS to minimise toxic bromate production. Moderate ozonation to an ORP of about 300-350 mV is common and considered as safe for marine fish in RAS, although some production of bromate has been demonstrated at this level (Tango and Gagnon, 2003). Ozonation to 350 mV do not represent efficient disinfection in a marine RAS (Hsieh *et al.*, 2002), but has been shown to generally improve water quality (Kobayashi *et al.*, 1993, Tango and Gagnon, 2003) leading to increased fish survival and growth (Ozawa *et al.*, 1991, Reid and Arnold, 1994).

Ozonation limits the build up of biologically inert compounds in the RAS by splitting complex organic substances to more biodegradable molecules that are more easily removed by heterotrophic bacteria (Paller and Lewis, 1988, Sugita *et al.*, 1996, Summerfelt, 2003). Sites of initial ozone reaction are multiple bonds or negatively charged atoms (Brazil *et al.*, 1998). Ozone may enhance fine solids removal by changing particle size and surface properties, which can make particles easier to settle (Rueter and Johnson, 1995), filter out or float. However, Krumins *et al.*, (2001) found that under normal circumstances in a RAS, ozone addition does not affect the particle size distribution, even if it increases particle removal from the culture water. Addition of ozone may increase the efficiency of the protein skimmer and in this way reduce the organic, particle and microbial load on the system (Sander and Rosenthal, 1975). The improvement of the skimming process is a result of the increased fraction of charged molecules in the water, which leads to more particles binding to the surfactants in the foam. However, the effect of ozone on particles is variable and in some cases ozone may increase particle stability and decrease flocculation (Edwards and Benjamin, 1991, Wilczak *et al.*, 1992). Little is known about the effects of the ORP on the microbial community or the larvae in their natural habitat.

UV-irradiation
Ultraviolet light (UV) is electromagnetic radiation with a wavelength of 100-400 nm, divided into UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm). UV-C is the UV radiation with highest energy. UV light can be artificially produced with electrical discharges in lamps containing mercuric vapour. Mercuric vapour emits most of its UV radiation as monochromatic light with a wavelength of 254 nm, which has high inactivation efficiency on microorganisms. UV rays in this spectral region are absorbed by DNA molecules. This may lead to splitting of the DNA strands and the covalent dimerisation of adjacent thymine molecules, with inhibition of replication as a consequence. The effect can be temporal or lethal depending on the repair mechanisms and the degree of UV resistance of the cell. Two important repair mechanisms are photoreactivation (Liltved and Landfald, 1996, 2000) and excision repair. Excision repair is based on the recognition and removal of a short distorted DNA segment leaving a single-strand gap in the DNA that is subsequently filled in by DNA polymerase with the undamaged strand as a template. Photoreactivation is attributed to the ability of light in the wavelength range of 330-480 nm to activate repair enzymes (DNA photolyase) which split the dimers without excising the distorted region. The rate of photoreactivation is strongly correlated with light intensity, whereas excision repair may be carried out in the dark.

The UV irradiation dose is the product of radiation intensity (amount of energy per surface unit) and irradiation time. The intensity of UV irradiation decreases with travel length through water due to attenuation and dissipation. Severe reduction in the efficiency of UV systems may occur due to turbidity of the water which protects microorganisms from the UV by particle shading (Liltved and Cripps, 1999, Paper 4). UV is also absorbed by other molecules such as oxygen and DOM to produce reactive compounds in the water that may affect marine larvae. UV irradiation has been found to indirectly cause an elevated occurrence of cataract (opaqueness of the eye lens) in juvenile cod in a RAS. The mechanism was suggested to be the production of ozone or another photoproduct formed by the radiation causing oxidative damage to lens tissue (Björnsson, 2004). According to Björnsson (2004), the UV treated seawater rapidly lost the potential to develop cataract in cod. The half-life of the effect was reported to be about 1 minute.

1.2.8 Microbial environment

Production of a high number of juveniles of good quality is still the bottleneck in the expanding production of marine cold water fish. Common challenges in the early life stages are a generally poor performance of fish larvae and a highly varying production with poor reproducibility between replicate tanks. The trouble is manifested in problems with initiating feeding, slow growth and incidents of arrested development and abrupt mortality. Complex factors are expected to influence the performance of fish larvae, including nutritional and genetic factors, egg quality, levels of waste products in the water, variability of the physiochemical environment and microbial interactions. However, the lack of reproducibility can occur even with the same feed and full sibling groups, and can then hardly be explained by nutrition, genes or egg quality. The observed variation have been hypothesised to be attributed mainly to detrimental microbial relationships (Vadstein *et al*., 1993, 2004, Vadstein 1997), which has been supported in studies using antibiotics (Munro *et al*., 1994, Skjermo *et al*., 1997, Verner-Jeffreys *et al*., 2004) and microbial maturation of water, defined as the selective promotion of K-strategic bacteria (Skjermo *et al*., 1997, Salvesen *et al*., 1999, Skjermo and Vadstein, 1999). The first days following hatching, the larvae are sensitive and in a critical stage for microbial colonization of gut and skin. The microbial community of the water in intensive aquaculture is often destabilised by disinfection and addition of organic matter, which increase the possibilities for invasion by opportunistic types of bacteria. There is consequently a need for functional microbial control strategies in the time interval between the hatching of larvae and the completion of metamorphosis. As discussed by Vadstein *et al*. (1993, 2004) the effect of microbial mitigation is expected to be greatest when fish are stressed or when performance is compromised by other factors.

Marine hatcheries show important differences from ongrowing fish farms in the levels of biomass and waste production and in how vulnerable the fish are. In hatcheries waste products may not be a problem during the live feed stage (Olivar *et al.*, 2000), but stability and control of the bacterial environment are paramount. At some point down the production line, fish become more robust and addressing other challenges than microbial control become increasingly more important, i.e. oxygen demand and waste removal.

1.3 Interactions between microbes and fish larvae

The reader is referred to Hansen and Olafsen (1999) for a comprehensive review on the bacterial interactions in early life stages of marine cold water fish. The type of bacteria present, the composition of the microbial community and the total amount of bacteria are factors that may influence the rearing conditions of the larvae. The amount and types of bacteria that larvae are first exposed to may be especially important, as it has been shown to

be harder to influence the microbial flora of the skin and digestive tract of individual larvae once colonized (Ringø and Vadstein, 1998). Bacterial densities can reach very high numbers in larval rearing units ($>10^8$ cells mL^{-1}) (Vadstein et al., 1993). A pronounced variation in the composition of the microbial flora has been found between individual cod larvae from the same tank (Fjellheim et al., submitted). High inter-individual variation in microbiota is also common in other organisms, like humans (Booijink et al., 2010). The variation in gut microbiota may influence the development of individual phenotypes, as has been shown for rodents (Holmes and Nicholson, 2005).

The microbial flora in aquaculture systems include both autotrophic (e.g. nitrifiers) and heterotrophic bacteria. Heterotrophic bacteria consume oxygen and organic matter. Some of the heterotrophic species are obligate or facultative pathogens that may cause disease in fish. Specific pathogens have been identified for marine fish larvae, like *Vibrio anguillarum* (Sandlund and Bergh, 2008, Sandlund et al., 2010). However, the route to infection in larvae is shaped by the lack of a fully developed specific immune system, and specific pathogens are generally not considered as a major problem in hatcheries (Vadstein et al., 1993, Vadstein, 1997). On the contrary, outbreaks of disease often occur in apparent absence of known pathogens, by way of latent infection carriers, and the majority of the diseases in marine fish larvae are thought to be caused by opportunistic microorganisms that become pathogenic when the host's resistance is lowered by environmental stress factors (Vadstein et al., 2004). Stress responses can be triggered from various influences in fish farms. Other types of heterotrophic bacteria may be important for the normal development of larval digestion and disease resistance, and may have a positive influence on the performance of the fish larvae (Fjellheim, 2006). Neutral microbes present in the water neither help nor harm the fish larvae directly, but may in an ecological sense contribute to a good microbial water quality by occupying niches and preventing proliferation of harmful species.

The carrying capacity (CC) is the maximum number of bacteria that can be sustained in the system over time. Dissolved organic matter (DOM) is typically the growth limiting factor defining CC for heterotrophic bacteria. The microbial state of the rearing water depends on the supply of bacteria and organic matter, together with selective forces in the tank and in the water sources. The main contributors of bacteria to marine hatchery tanks are live feed (Skjermo and Vadstein, 1993, Blancheton and Canaguier, 1995, Olsen et al., 1999, Makridis et al. 2000a,b), algae (Salvesen et al., 2000) and intake water. Live feed are commonly associated with high levels of opportunistic bacteria including *Vibrio* spp. and haemolytic types (Skjermo and Vadstein 1993, Salvesen et al., 1999, Olsen et al., 2000). The bacterial flora of the live feed is shown to be directly transferred to the start feeding larvae (Olsen et al., 2000). A correlation has been found between numbers of bacteria on the live feed and larval mortality (Munro et al., 1999). Blancheton and Canaguier (1995) found that during the live feed period, bacterial production of RAS rearing tanks depended on the concentration of food in the tank, and was higher than the bacterial production of the biofilter. Even if colonization may be selective, rearing water is an important first source of bacteria, as marine larvae actively take up bacteria (and algae) from the water even before ingesting feed (Reitan et al., 1998).

The composition of the microbial community seems to be more important for larval performance than absolute bacteria numbers as long as numbers are not extreme (Munro et al., 1995, Salvesen et al., 1999, 2000, Verner-Jeffereys et al., 2004). Early exposure to relatively high bacterial densities may be important for immune tolerance development (Davina et al., 1982, Rombout and van den Berg, 1985, as cited by Hansen and Olafsen, 1999). In addition,

stable high levels of bacteria may typically include a relatively low amount of opportunists, which has been suggested to be a result of the opportunistic bacteria being less efficient competitors in crowded environments (Salvesen *et al.*, 2000). Consequently, bacterial abundance should not be used as an unconditional marker of the quality of the microbial environment.

It is difficult in practice to assess the microbial environment in relation to the fish larvae because of the complexity of the system that includes a great number of different species. Even though this field of research is developing rapidly, there are methodical limitations to describe complex microbial communities in great detail. Fortunately, in many cases a general division in microbes that are good, bad or neutral for the larvae suffice. Vadstein *et al.* (1993) used a number of criteria based on the ecological theory of r/K-selection (MacArthur and Wilson, 1967) to describe the state of microbial maturity of rearing water. According to the theory, selective pressures drive succession in one of two generalized directions: r-selection occurs in unstable or unpredictable environments where the ability to reproduce quickly is crucial, whereas K-selection occurs in stable or predictable environments where the ability to compete successfully for limited resources (e.g. DOM) is more important, i.e. in a community close to CC. K-strategists are strong competitors in crowded environments with a low substrate supply per capita, whereas r-strategists grow fast in environments with empty niches and high resource availability. Generally, an un-mature community with a high share of fast-growing r-strategic bacteria is believed to be detrimental for fish larvae (Vadstein *et al.*, 1993). Further, an unstable microbial community with low diversity is considered to be hazardous, as it promotes r-selection. The classification of bacteria along an r-K axis is a useful approach to a functional description of the microbial community in relation to marine larvae when information about beneficial probiotic candidates or specific pathogens is less important and difficult to obtain. Bacteria prone to cause general infections are accounted for, as well as the group of microbes which are less likely to influence larvae adversely. In addition it is a useful concept to understand the ecological succession in the microbial community when exposed to perturbations. The practical division in r- and K-strategists may be based on the relative maximum specific growth rates of the bacteria (Skjermo *et al.*, 1997, Salvesen and Vadstein, 2000).

1.4 Effect of intensive fish cultivation procedures on the microbial community of the rearing water

The ocean, the natural habitat of the fish larvae, is characterised by low and stable levels of organic matter and bacteria. Current production routines of intensive aquaculture include modifications and frequent perturbations of the microbial community in the water. Seawater is pumped to the land based fish farm where it is commonly filtered, heated, aerated and disinfected. High and fluctuating concentrations of organic matter are distributed to the rearing tanks which are stocked with high densities of larvae. In addition, the live feed supply the rearing water with bacteria several times daily. Disinfection of intake water is a necessary barrier against pathogens, but represents a major perturbation to the microbial community by diminishing competition and making organic matter, i.e. dead biomass and oxidized organic compounds, more available for degradation (Hess-Erga, 2010, Hess-Erga *et al.*, 2010). Addition of organic matter increases the microbial CC of the system, leaving room for more bacteria. Following a perturbation, like disinfection or a sudden increase in the concentration of organic matter, the microbial community of a water body will develop over time. Primary colonisers are typically opportunistic r-strategists with higher reproductive rates (Hess-Erga *et al.*, 2010). Given time, a succession of increasingly competitive species follows as the availability of resources like bacteria substrate change, i.e. the r-strategists are gradually

replaced by K-strategic bacteria. At the same time, the total number of bacteria rises to the (new) CC in an exponential manner. The undesirable result of current intake water treatment and production routines of intensive hatcheries is an unstable microbial environment with low diversity and the selection for opportunists, including pathogens and unfavourable species.

2 Objective

Marine hatcheries are complex systems involving several organisms kept at high densities, high nutrient content and a physiochemical environment in change due to activity of the organisms and system operation. A highly variable performance of the larvae in intensive culture has been attributed to detrimental host/microbe relationships (Vadstein *et al*., 1993, 2004, Skjermo *et al*., 1997, Salvesen *et al*., 1999). Intensive fish cultivation procedures tend to destabilise the microbial community which promote r-selection that may result in the domination of potentially harmful species (Hess-Erga *et al*., 2010).

The objective of this thesis was to suggest and explore simple and functional methods of water treatment to increase the control and improve the general microbial environment in the rearing water of marine larvae in intensive production of juveniles. Atlantic cod (*Gadus morhua* L.) was used as the model species. I have focused on the live feed period, which is characterised by highly sensitive and quickly developing larvae, low biomass and limited waste production. An important part of the motivation for my work was the development of practical methods that could easily be applied by the industry.

3 Strategies to increase control and improve the microbial environment in the rearing tanks

Disinfection is commonly used as a barrier against pathogens in the intake water and to combat very high numbers of bacteria in RAS. To investigate practical solutions of using water treatment to gain microbial control in marine hatcheries we looked into the inactivation of marine heterotrophic bacteria by UV-irradiation or ozonation in the presence of biotic and abiotic particles (Paper 4). Generally, the water in RAS that is subject to disinfection has different properties than clear intake water in FTS. The relation between particles, microbiota and disinfection method may affect the disinfection efficiency and the subsequent development of the microbial community in the rearing tanks of a RAS and a FTS differently (Paper 3 and 4). Although disinfection may be efficient against the introduction of specific pathogens in clear intake water, it is a non-permanent solution that in the longer term destabilise the microbial community (Hess-Erga *et al*., 2010). To address the problem of an unstable microbial community dominated by opportunists more sustainable methods than disinfection are obviously needed.

According to Vadstein *et al*. (1993) there are several approaches to increase the stabilisation of the microbial environment in the rearing water of marine hatcheries, and the elements and methods that were suggested in a strategy for microbial control are summarised in Figure 1 and Table 1. Several of the methods suggested are relevant for controlling the microbial community in the rearing water, including non-selective reduction of bacteria through reduced input and removal of organic matter used in Paper 3 and the selective enhancement of bacteria through selection of desirable bacteria that was used in Paper 1 and 2.
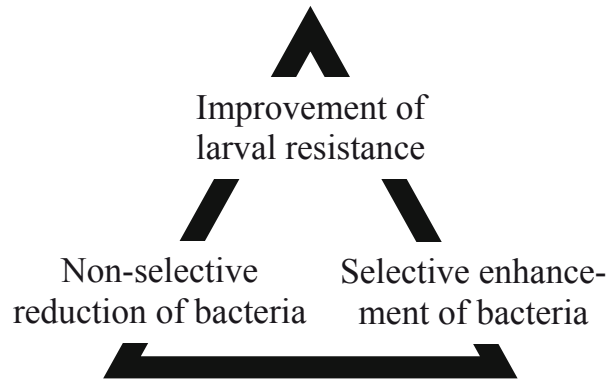
Fig. 1: Outline of the three elements in a strategy to obtain microbial control in the rearing of marine larvae (reproduced from Vadstein *et al*. (2004) by courtesy of Olav Vadstein).

Table 1: Examples of possible methods within the different elements in a strategy to obtain microbial control in the rearing of marine larvae (reproduced from Vadstein *et al*. (2004) by courtesy of Olav Vadstein).

1. Non-selective reduction of microbes:
a) Surface disinfection of eggs
b) Reduction in input of organic matter
c) Removal of organic matter
d) Grazer control of bacterial biomass
2. Selective enhancement of microbes:
a) Selection for desirable bacteria
b) Addition of selected bacteria to tanks
c) Incorporation of selected bacteria in feed
3. Improvement of resistance against microbes:
a) Stimulation of general immune system
b) Stimulation of secific immune system (vaccination)
c) Modulation of general and specific maternal immunity
d) Nutritional supplements improving susceptibility to microbes and wound healing

To improve the microbial environment of the larvae the fraction of opportunistic bacteria, including potential pathogens, should be reduced and the general diversity and the amount of beneficial bacteria types, including probiotic species and K-strategists, should be increased. Selective enhancement of bacteria in intensive rearing of larvae may involve two functionally different approaches: the addition of beneficial bacteria to water or feed (probiotica concept), or directional selection to reduce the fraction of potentially harmful bacteria. Whereas Fjellheim (2006) explored the probiotic approach, this thesis focuses on the use of selection to improve the microbial conditions for cod larvae.

The use of molecular methods to identify bacterial species has revealed that the microbiota of healthy organisms is more diverse than earlier assumed (Malinen *et al*., 2005) and that it may

vary greatly between individuals (Booijink *et al*., 2010, Fjellheim *et al*., submitted). Directional, community level selection is a general approach that recognises the complexity of the microbial community. The probiotic strategy, on the other hand, requires knowledge about the types of bacteria or combinations of bacteria that are beneficial, which creates the need for classification and characterisation of specific species. Per definition, the addition of probiotics reduces diversity, whereas K-selection increases it. It is also a challenge to make the probiotics remain in the systems, whereas directional selection favours the stable presence of the selected microbial composition. The addition of probiotics used in combination with directional selection is an unexplored strategy that could turn out to be favourable.

Disinfection and antibiotics reduce the number of competing bacteria without decreasing CC, destabilising the microbial community by increasing the amount of available substrate per bacteria, favouring the subsequent proliferation of r-strategists. The imbalance created by hygienic barriers may be counteracted by controlled recolonisation of the rearing water by stimulating microbial succession and promoting a more K-selecting environment. K-selection increases the microbial diversity and stability and reduces the fraction of opportunists. Directional selection towards a climax community close to CC requires microbial competition. In microbial maturation of water a K-selective pressure is created by reducing the nutrient supply per bacteria (Vadstein *et al.*, 1993). The microbial community is allowed to adjust to CC in a heterotrophic biofilter occupied by K-strategists (Skjermo *et al.,* 1997).The biofilter provides a large surface area for biofilm growth. A matured heterotrophic biofilter is stable and robust and operates close to CC of the incoming water. Microbial maturation of intake water has been shown to increase growth and survival of marine fish larvae (Skjermo *et al*., 1997, Salvesen *et al*., 1999). Microbial maturation is a well documented "black box" approach with few or no bacterial species characterised in the experiments. However, it is directed to control the microbial composition of the intake water before it is presented to the fish larvae. Because microbial maturation is only stable for the conditions under which it has been developed, any significant changes away from defining levels of for example nutrient concentration, space or oxygen may make the succession start over.

In most cases microbial maturation of intake water is adjusted to a relatively low CC compared to that found in the fish tanks. Hatching remnants, mortality, defecation, algae and live feed contributes significantly to the organic load (CC) on the rearing water, increasing the nutrient supply per bacteria. A higher CC in the fish tanks compared to the matured intake water provides resources for recolonisation and opens for opportunistic proliferation. Water with a bacterial community selected for at a CC closer to the levels in the enriched rearing tanks should hypothetically maintain higher microbial stability and limit the regrowth of bacteria. By maturing the water and eliminating the gap in CC between production steps, the fish farmer may gain microbial control. Realizing this, Salvesen *et a*l. (1999) encouraged the development of systems with capacity to produce microbially matured water at a CC matching that of the rearing tanks (see below). Another strategy may be to reduce CC in the rearing tanks to match that of the matured intake water. One method to maintain low CC in the rearing tanks may be to increase the water exchange rate; however, this also increases the rate of loss of the expensive live feed. Another approach may be to replace microalgae with inorganic clay in the rearing tanks to reduce the supply and increase the removal of organic matter (Paper 3), combining several elements of the strategy for microbial control (Table 1).

There are practically two ways to increase the CC of the matured water going to the tanks. One approach is to add organic matter to the maturing unit (biofilter) in a FTS. Another solution is to reuse the waste water from the rearing tanks to feed a heterotrophic biofilter

with organic matter in a RAS. Of these approaches, the use of RAS is the simplest way to feed the biofilter with a quantitatively and qualitatively similar mix of nutrients to that in the fish tanks. In addition it is clearly a better resource management as feed is not "wasted" on the biofilter. In a RAS the biofilter consumes organic matter from the fish and supply the fish tanks with bacteria. It may also be an advantage that RAS operation secures an extended time for water maturation in the system, provided the use of disinfection is limited. The drawback of using the RAS approach compared to feeding a biofilter in a FTS is the accumulation of waste products in the RAS. However, because of the low concentration of biomass the waste production is low during the first weeks post hatching and may generally be maintained at an acceptable level in a RAS during the live feed period (Olivar *et al.*, 2000). When the level of waste products starts to become problematic, the immune system of the larvae is more developed.

RAS are widely used to stabilise physiochemical water quality, reduce water consumption and control waste emission. On the basis of the theoretical deduction above it is hypothesised that RAS may also promote K-selection and stabilisation of the microbial community that can be advantageous for marine fish larvae during the live feed period (Attramadal *et al.*, 2010, Paper 1). The hypothesis predicts that the stabilising and maturing mechanism will be present in all RAS, without being designed or operated specifically for the purpose. However, disinfection may destabilise the system and interfere with the maturing effect because it reduces the amount of bacteria competing for substrate without reducing CC, promoting regrowth in the rearing tanks (Paper 2).

4 Approach and experimental aspects

On the basis of the described strategies to increase microbial control in the rearing water of cod larvae, attempts were done to develop simple and functional methods for the live feed period of larval rearing that could easily be applied by the industry. These methods were tested under conditions and at a scale that could be compared to industrial systems. Two different approaches were investigated, the hypothetical reduction and removal of organic matter by substitution of microalgae with clay (Paper 3), and the hypothetical maturation and stabilisation of the microbial community in a RAS compared to a FTS (Paper 1 and 2). In addition, the effects of disinfection on the microbial maturation and stabilisation in RAS (Paper 2) and in relation to inorganic and organic particles in the water (Paper 4) were evaluated.

In the first feeding experiments (Paper 1, 2 and 3) information was collected to describe the general physiochemical environment, the state of microbial maturation and stability of the systems and the performance of the larvae (growth, survival and the ability to cope during weaning to dry feed). In Paper 3 the effects of the addition of clay on the concentration of organic matter and live feed in the rearing water was compared to rearing regimes with live algae (*Isochrysis galbana*) and algae paste (*Nannochloropsis oculata*). Figure 2 shows (a) the RAS that was used in Paper 1 and (b) the FTS, RAS UV and RAS O_3 that were used in Paper 2. In Paper 4 the UV irradiation or ozone dose-dependent inactivation of free living and biotic particle (rotifer) associated bacteria and the protective effect of biotic and abiotic (clay) particles as a function of particle concentration was investigated.
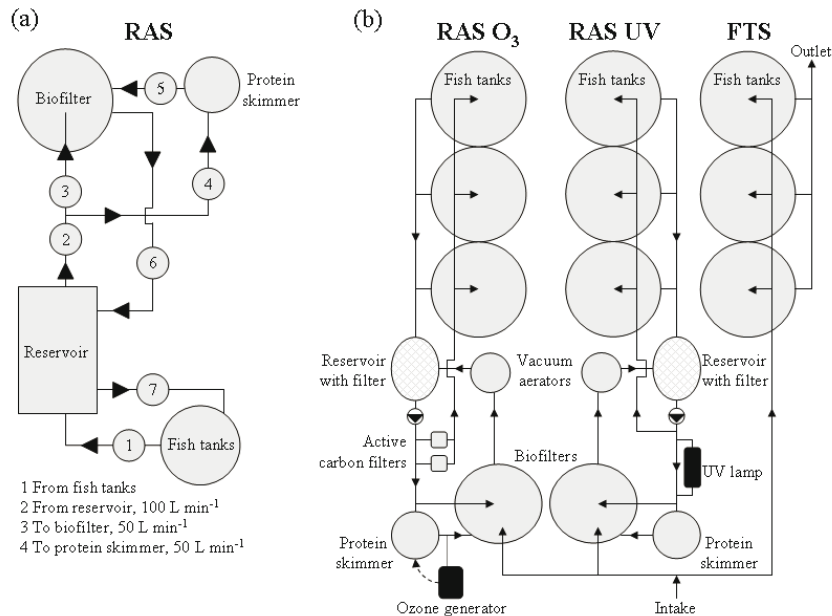
Fig. 2: Schematic drawing of (a) the RAS used in Paper 1 and (b) the FTS, the RAS with UV irradiation (RAS UV) and the RAS with ozonation (RAS O₃) used in Paper 2 (not to scale).

4.1 Microbiological tools

4.1.1 Characteristic markers for a mature microbial community

At the moment there is not enough knowledge to suggest the composition of a malign and a benign microbiota of fish. The r/K-concept is a way to circumvent this. The classification of the community of bacteria in the rearing water along an r-K axis may indicate the relative presence of opportunistic bacteria that may be harmful for the fish. Characteristic markers for K-selected, mature communities in water include a more stable and resilient composition of the microbial community over time, higher species richness and diversity and a lower fraction of opportunists (Vadstein *et al.*, 1993, Salvesen, 1999). Higher stability may be related to less change in the species composition over time. Similarly, higher reproducibility in the microbial community composition may be defined as less variability between replicate rearing tanks. Because r-strategic opportunists are characterised by high maximum growth rates in contrast to K-strategic specialists, the fraction of fast-growing bacteria may be used as a measure of the relative abundance of opportunistic bacteria (Skjermo *et al.*, 1997, Salvesen and Vadstein, 2000). For the results in this thesis, the term opportunistic (r-selected) bacteria is used to denote the colony forming units (CFU) emerging the first two days of incubation on M65 agar at 12°C, as described by Salvesen and Vadstein (2000). In Paper 3, visible colonies on TCBS plates were also counted after 2 days of incubation, thus the TCBS counts represent the opportunists that grow on this agar type. Several *Vibrio* species form colonies on TCBS agar

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(Randrianarivelo *et al*., 2010), but TCBS counts may also represent bacteria from other taxonomic groups (Lopez-Torres and Lizarraga-Partida, 2001).

An increase in the fraction of cultivable bacteria (CB) is common when substrate levels increase due to perturbations. At a similar CC, a low CB may suggest a high level of specialised bacteria with a narrow niche width characteristic of a more mature community (Skjermo *et al*., 1997). However, CB may be a less useful criterion to compare the relative maturation of systems with very different loads of substrate. For example, higher CB was observed in microbially matured water compared to filtered water during the *Artemia* period in an experiment with turbot larvae, *Scophthalmus maximus* (Salvesen *et al*., 1999). CB has been found to increase with increasing C/N ratio and from inlet to outlet in a marine biofilter (Michaud *et al*., 2006, 2009).

4.1.2 Methods to characterise activity and composition of complex microbial communities

Most marine bacteria that may be observed via microscope are not cultivable on traditional growth media (the great plate count anomaly, Staley and Konopka, 1985). Measurements of the production of bacterial biomass have long traditions in studies of natural aquatic environments and it generally reflects the activity of the heterotrophic bacteria, and not just that of the cultivable fraction. The incorporation rate of radioactively labeled leucine (Paper 2) can serve as an index of protein synthesis by bacterial assemblages in aquatic systems, because addition of extracellular leucine inhibits leucine biosynthesis and the molar fraction of leucine is constant in a wide variety of proteins, indicating that changes in leucine incorporation rates reflect changes in rates of protein synthesis rather than changes in the leucine content of proteins (Kirchman *et al*., 1985).

Molecular methods are useful to characterise the composition of complex microbial communities, as they are sensitive, rapid, specific, and without the need for cultivation and isolation of the bacteria. The most frequently used molecular marker for the investigation of microbial communities is the gene encoding the small subunit ribosomal RNA molecule, 16S rDNA. This gene occurs in all procaryotic organisms and includes enough sequence variability to distinguish between a wide range of species as well as highly conserved regions suitable for designing universal primers. In a polymerase chain reaction (PCR) the specific DNA sequence is amplified through repeated (thermal cycling) melting and enzymatic replication. To characterise complex bacterial communities, PCR-fragments may be analysed by fingerprinting techniques like denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al*., 1993, Marzorati *et al*., 2008) and terminal-restriction fragment length polymorphism (T-RFLP) (Liu *et al*., 1997, Osborn *et al*., 2000). The profiling of a microbial community by DGGE is based on the separation of similar sized PCR-fragments in a linearly increasing gradient of denaturant according to difference in nucleotide sequence. T-RFLP is based on the position of a restriction site closest to a labeled end of the amplified gene. PCR amplification for T-RFLP is performed with one or both primers having their 5' end labeled with a fluorescent molecule. The amplicons are digested by restriction enzymes before being separated in a DNA sequencer where the size of each of the individual terminal fragments is determined by a fluorescence detector. PCR-based DGGE and T-RFLP are subject to all the biases inherent in any PCR amplification approach (von Wintzingerode *et al*., 1997), and have a detection limit for species with a low frequency in the sample. PCR-DGGE has a reported sensitivity of approximately 1 % of the population (Muyzer *et al*., 1993). For both methods there is a probability for under-estimating species richness.

5 Results and general discussion

5.1 Reduction in input and increased removal of organic matter

The substitution of microalgae with clay to increase turbidity in the rearing tanks was suggested as an easy and cost efficient method to reduce the supply and increase the removal of organic matter to rearing tanks, with the goal to lower the CC and limit the proliferation of bacteria during first feeding (Attramadal *et al.*, 2007, Paper 3).

The addition of clay resulted in significantly reduced levels of bacterial substrate in the rearing water compared to rearing regimes with live algae or algae paste ($p < 0.001$, one-way ANOVA, Table 2). This was mainly a result of the reduced input of organic matter in the tanks added clay. The clay had a high surface area to weight ratio. The clay aggregated and co-precipitated organic matter from the rearing water. The sediments with the adsorbed organic matter could easily be removed by cleaning the bottom of the tanks. However, as the volume of the layer of debris on the bottom of tanks was relatively small compared to the total volume of the tank (~2-3 %), our estimate suggests that only 5 % of the total amount of organic matter in the tank could be removed with the sedimented clay each day during cleaning.

Table 2: The performance of the cod larvae and the characteristics of the microbial community and the organic matter level of water receiving live algae, algae paste or ceramic clay in the first feeding experiment (day 0-20 ph) in Paper 3 (average ± SE).

Treatment comparison day 0-20 ph	Live algae	Algae paste	Clay
Dissolved organic carbon (mg L^{-1})	3.3 ± 0.0	2.6 ± 0.0	1.8 ± 0.0
Particulate organic carbon (mg L^{-1})	0.9 ± 0.0	0.9 ± 0.1	0.1 ± 0.0
Total cell counts mL$^{-1} \times 10^6$	8 ± 1	12 ± 1	6 ± 1
CFU mL$^{-1} \times 10^5$	2 ± 0	16 ± 3	2 ± 0
Opportunistic bacteria (%)	9 ± 7	23 ± 13	30 ± 5
TCBS counts (%)	0.1 ± 0.0	0.7 ± 0.2	0.5 ± 0.4
Cultivable bacteria (%)	2 ± 1	27 ± 11	4 ± 1
Larval growth (% SGR)	7.1 ± 0.5	6.3 ± 0.5	7.3 ± 0.5[a]
Larval survival (%)	36 ± 2	22 ± 3	50 ± 3[a]

[a]Not included the replicate tank with total mortality 13 dph

A lower abundance of bacteria in the rearing water of the tanks receiving clay reflected the reduced levels of bacterial substrate ($p < 0.001$). This is in accordance with results reported by Björnsdóttir (2010). Rearing tanks receiving algae paste showed higher abundance of bacteria and a higher share of cultivable bacteria and TCBS counts than tanks receiving clay or live algae (Table 2). Although the general abundance and cultivability of bacteria was reduced in the tanks with clay compared to tanks receiving algae paste, there was an increase in the fraction of TCBS counts and opportunists compared to tanks added live algae. In our experiments with clay the intake water was not matured before bacterial substrate, rotifers and larvae were introduced. According to the rationale explained above (see *4. Approach and experimental aspects*) a combination of microbial maturation of intake water and clay addition in tanks (lowering CC to the level of the intake water) could be a way to gain

microbial control and increase stability in FTS for marine larvae. Tanks with live *I. galbana* maintained low numbers of bacteria and very low shares of opportunistic bacteria and TCBS counts in both water and rotifers despite the higher levels of organic matter, which may be due to the presence of antibacterial compounds. Addition of *Isochrysis* sp. has been found to inhibit proliferation of opportunists in the rearing water of turbot (*Scophthalmus maximus*) (Salvesen *et al.*, 1999).

Rotifers and fish larvae did not seem to be adversely affected by the clay in our experiments. On the contrary, the performance of larvae in tanks added clay was good (Table 2). Cod larvae in tanks with clay or live algae initiated exponential growth earlier than larvae in tanks receiving algae paste. Early initiation of exponential growth is considered to reflect high quality of marine larvae (Reitan *et al.*, 1993, Skjermo *et al.*, 1997). The Larvae in clay tanks had significantly higher dry weight than larvae in tanks receiving algae paste at day 5 and 20 post hatching ($p = 0.016$ and 0.025, respectively). One replicate of the tanks receiving clay showed total larvae mortality on day 13 ph, which may be connected to a markedly higher concentration of TCBS counts in that tank 3 days post hatching (dph). In the remaining replicates with clay, fish larvae showed a higher survival than in any tank of the other two treatments. The tanks receiving algae paste were generally considered to have an inferior microbial environment compared to the other two treatments.

Only one type of ceramic clay was studied in this thesis, selected on the basis of being used in the industrial aquaculture of halibut. Different types of clay have different properties with respect to level of purity, particle size, cation exchange capacity, adsorption capacity and swelling ability which may affect the fish, the light conditions, the bacteria and the aggregation of organic matter. Which is the optimal type of clay for this application is yet to be evaluated. Little is also known about the effects of clay on the nutrition and development of larvae.

In all the first feeding experiments presented (Paper 1, 2 and 3), the best performance of fish have been observed in the treatments that were thought to offer the highest turbidity during the rotifer phase. Although it was in a similar range in all of the treatments, systematic differences in the level and/or source (algae cells or clay) of turbidity may have had an effect on the performance of the larvae.

Conclusion - Reduction in input and increased removal of organic matter
One strategy that may be used to control the microbial environment in the rearing water is non-selective reduction of bacteria by reducing the supply and increase the removal of organic matter. Substituting algae paste with clay appeared to limit high abundance of bacteria by reducing the load of organic matter, and hence reducing the microbial CC in the rearing tanks, at a stage when the effect of external water treatment was limited. Clay addition is cheaper and simpler than application of both algae paste and live algae, and it seems to be a good alternative in terms of cost and performance in the early stages of the production of cod.

5.2 Recirculation as a microbial control strategy
In Paper 1 it was hypothesised that RAS have the potential to stabilise and mature the microbial community of the water through 1. the promotion of K-selection and 2. securing no difference in the CC of in-flowing and tank water. Properties of RAS that have been suggested to contribute to microbial stabilisation include long water retention time, a large surface area of the biofilter for bacterial growth and the stable high CC throughout the system.

In two first feeding experiments the development of the microbial community and the performance of larval cod in RAS were compared to that of FTS (Paper 1 and 2). The stability and maturity of the microbial community was studied (see 4.1.1 *Characteristic markers for a mature microbial community*).

The microbial community composition in the RAS tanks apparently developed differently from that in the FTS tanks in both experiments despite significant contributions of bacteria from the common feed and algae. This emphasises that selection by water treatment is a significant force. Water treatment regime explained most of the variation in microbial composition during the live feed period, according to principal component analysis (PCA) of bacterial community composition of the rearing water based on terminal-restriction fragment length polymorphism (T-RFLP) of PCR amplified bacterial DNA of the 16S RNA gene. Figure 3 shows the arithmetic mean of the PCA score values for each feed type period, the standard deviation (SD) and the trajectories of the different systems in the two experiments. There was a general difference in microbial composition between the systems (PC1) and a similar development over time or with change of feed type (PC2). PC1 accounted for 45 % and 36 % of the variation in the data set in Paper 1 and 2, respectively, and PC2 accounted for 17 % in both experiments. The $SD_{PC1} \times SD_{PC2}$ value represents the amount of variation in microbial community composition in the systems. In Paper 1 the RAS showed a more stable bacterial flora with an $SD_{PC1} \times SD_{PC2}$ of 0.02 compared to 0.05 for the FTS. In Paper 2 the FTS showed the most unstable composition of bacteria with an $SD_{PC1} \times SD_{PC2}$ of 0.014 compared to 0.006 and 0.002 for the RAS UV and RAS O_3, respectively. In both experiments, the higher variation in microbial community composition in the FTS was mainly related to the water treatment (high SD_{PC1}).
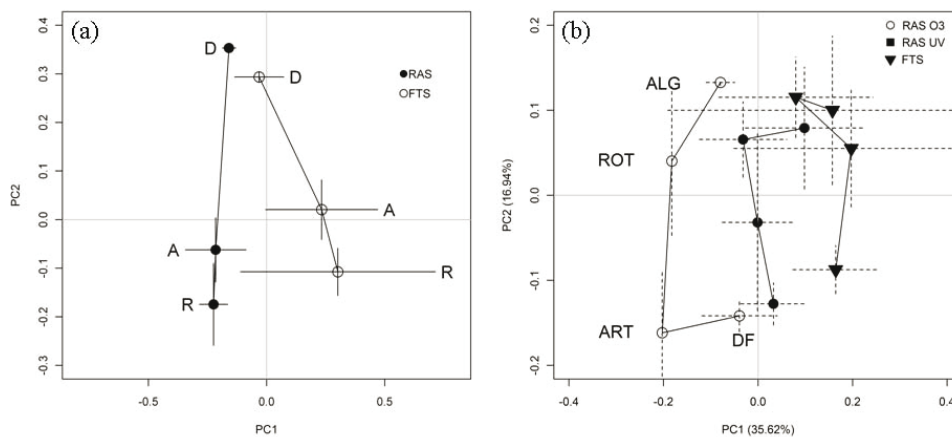
Fig. 3: PCA of the data on bacterial community composition of the water in the first feeding experiments based on PCR/T-RFLP fingerprinting in (a) Paper 1 and (b) Paper 2. Letters indicate different feeding regimes: ALG = algae period R/ROT = rotifer period, A/ART = *Artemia* period, D/DF = dry feed period.

Characteristics of the microbial community of the rearing water in the different systems in Paper 1 and 2 are summarised in Table 3. Generally, a higher abundance of bacteria was found in water of RAS than of FTS. In Experiment 1 the fraction of opportunistic bacteria was higher in the FTS than in the RAS during the rotifer period. In addition, the diversity of bacteria was lower and less stable in the FTS than in the RAS.

According to the criteria used to define a more stable and K-selected microbial community, the RAS with moderate ozonation generally offered a more beneficial microbial environment for the larvae than the FTS in both experiments. The overall trends supported the hypothesised development of a more even, stable and diverse structure of the microbial community in a RAS. Significantly different and presumably more beneficial microbial communities in RAS compared to FTS have also been described in other publications both in water (Verner-Jeffreys et al., 2004) and in larvae (Fjellheim et al., 2007, Fjellheim et al., submitted). The elaborate work to reveal the specific species and functional groups associated with RAS operation should be continued to optimise RAS for microbial control in marine hatcheries.

Table 3: Characteristics of stability and maturity of the microbial communities of the water in the first feeding experiments in Paper 1 and 2 (average ± SE). The total bacteria cell counts were done via microscopy. The fast growing opportunists are shown as relative fractions of total CFU. Cultivability is the proportion of CFU of the total bacteria cell counts. Shannon's diversity index (H') was calculated for the T-RFLP profile of each sample, using the abundance of operational taxonomic units (OTU) for species richness. The interpretation of the abundance of OTU as species richness is a simplification that may not be valid for the actual population, as the total abundance of species is unknown, but was used to give some idea of the relative distribution and evenness of the dominating species of bacteria (Blackwood et al., 2007).

Period	Rotifer					Artemia				
Experiment	1		2			1		2		
System	RAS	FTS	RAS UV	RAS O$_3$	FTS	RAS	FTS	RAS UV	RAS O$_3$	FTS
Total counts of bacteria × 10^6 mL^{-1}	6.3 ± 1.0	4.4 ± 1.2	3.0 ± 0.3	2.9 ± 0.4	1.0 ± 0.1	6.3 ± 0.6	1.1 ± 0.2	5.7 ± 0.8	9.9 ± 1.9	1.7 ± 0.2
Colony forming units ×10^6 mL^{-1}	1.1 ± 0.5	0.7 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	3.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.3	1.9 ± 0.2	2.0 ± 0.7
Cultivability (CB)	17 ± 4	12 ± 1	20 ± 4	28 ± 8	34 ± 9	47 ± 3	22 ± 7	2 ± 0	3 ± 1	11 ± 3
Opportunists (%)	18 ± 8	27 ± 7	4 ± 1	4 ± 1	7 ± 3	24 ± 8	45 ± 5	41 ± 10	14 ± 7	7 ± 2
Shannon's diversity index	1.7 ± 0.1	1.2 ± 0.4	2.8 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	1.9 ± 0.2	1.2 ± 0.2	2.9 ± 0.2	2.8 ± 0.4	2.7 ± 0.2
Species richness	9 ± 1	6 ± 2	44 ± 5	49 ± 4	42 ± 4	14 ± 4	6 ± 1	47 ± 7	55 ± 8	29 ± 3

Complex factors are expected to influence the performance of fish, including levels of waste products, variability of the physiochemical environment as well as the level of turbidity and the microbial composition. RAS operation led to waste accumulation, but apparently stabilised the microbial environment enough to counteract possible negative effects of waste accumulation. Young larvae are sensitive to microbial infections and may especially benefit from microbial control and stability (Vadstein et al., 1993, Skjermo et al., 1997, Salvesen et al., 1999). In both experiments (Paper 1 and 2) cod larvae reared in the RAS performed equally well or better than their FTS siblings despite being exposed to an apparent inferior physiochemical water quality. The amount of algae paste (Paper 1) or live algae (Paper 2) added to the RAS tanks was the same as for the FTS tanks, which, as a result of reuse, led to higher turbidity in the RAS. For some reason the protein skimmer more efficiently removed POC from the RAS UV than the RAS O$_3$ in Paper 2, which may have caused higher turbidity in the RAS O$_3$. It is possible that the higher turbidity was positive for the performance of the larvae, and this should be investigated further in the future to separate it from the microbial effect on the fish. The RAS UV in Paper 2 resembled the FTS most with respect to microbial composition and the performance of larvae. This may be related to the disinfection efficiency of the UV-system as discussed below (see *3.3.2 The type of hygienic barrier and/or the disinfection efficiency used in RAS affect the development of the microbial community of the rearing water*). The possibility that moderate ozonation in itself may have a beneficial effect on the microbial community development related to the fish or on the larvae directly cannot

be ruled out from the presented results. Figure 4 shows the dry weight and survival of the cod larvae after the live feed period (30 dph) in the replicate tanks of the FTS and the RAS in Paper 1 and 2.

Although all systems had an acceptable physiochemical water quality during the live feed period, RAS rearing water always contained more nitrogenous waste, particles and dissolved organic matter. In both experiments pH decreased in the RAS during the *Artemia* period, reflecting higher CO_2 levels and nitrification. A similar pattern of decreased physiochemical water quality as a function of water reuse was observed in a study of juvenile cod (Foss *et al*., 2006). The physiochemical water quality quickly deteriorated during the dry feed period in the RAS to the extent that this period may be considered a stress test on the larvae. The physiochemical water quality was particularly bad in the two RAS in Paper 2 during the dry feed period. The main reason for this was that the systems were under-dimensioned, with too low water exchange rates. In both experiments the physiochemical water quality in the FTS was also reduced during the dry feed period, but not to the same extent.

In both experiments the ammonia to nitrite conversion step seemed to work satisfyingly in the RAS. However, an accumulation of nitrite indicated a slower establishment and response of the nitrite to nitrate conversion step, as typically seen for marine biofilters (Manthe and Malone, 1987). The nitrite oxidation reaction step was not absent, however, as an accumulation of nitrate was observed in both experiments. Levels of un-ionised ammonia and nitrite were considered safe, although the level of nitrite may have been suboptimal for the larvae in the RAS during the dry feed period (Poxton and Allouse, 1982, Siikavuopio and Sæther, 2006). Especially the RAS UV in Paper 2 showed high concentrations of nitrite during the last part of the dry period, which may have stressed the larvae. Ozone oxidation of nitrite to nitrate (Rosenthal and Otte, 1979, Borges *et al*., 2003) may have contributed to the lower nitrite levels in the RAS O_3 compared to the levels in the RAS UV.

In Paper 1 RAS larvae started to grow earlier than the larvae in the FTS (significantly higher DW larvae^{-1} on 5 dph, p = 0.002, Kruskal-Wallis and Mann Whitney U-test). In Paper 2 the RAS O_3 larvae showed significantly higher DW than the larvae in the RAS UV on days 17, 27, 30 and 37 post hatching (p < 0.035 one-way ANOVA and t-test), and significantly higher DW than the FTS larvae on days 27, 30, 37 and 45 post hatching (p < 0.003).

On average, the survival through the live feed period was higher in the RAS (n = 9) than in the FTS (n = 4) in Paper 1. In Paper 2 one of the replicate RAS O_3 tanks stood out with its low survival during the live feed period, whereas the other two replicates showed the highest survival of the experiment. From day 16 ph the density of larvae estimated by photography was significantly higher in the RAS O_3 than in the RAS UV and FTS (p = 0.017).

The RAS larvae apparently coped better during the stressful transition to dry feed even if the physiochemical water quality was considered to be inferior in all of the RAS compared to the FTS. The RAS larvae in Paper 1 showed significantly higher survival through the dry feed period than FTS larvae (P < 0.001, Wilcoxon (Gehan) Statistics, life history analysis of survival). Survival was higher in the RAS O_3 than in the other treatments through the dry feed period in Paper 2 (p = 0.015). The FTS included the tank with the lowest survival of larvae through the experiment. In addition, the FTS larvae showed significantly lower DW than the larvae in the RAS UV and RAS O_3 at the end of the dry feed period in Paper 2 (45 dph, p < 0.001). The metamorphosis and the transition from live prey to formulated feed are often challenging periods in hatcheries (Sahin 2001, Ustundag *et al*., 2002). The better performance

of RAS larvae than FTS larvae facing this challenging period indicates superior quality, which may reflect better conditions in the RAS the first days after hatching.
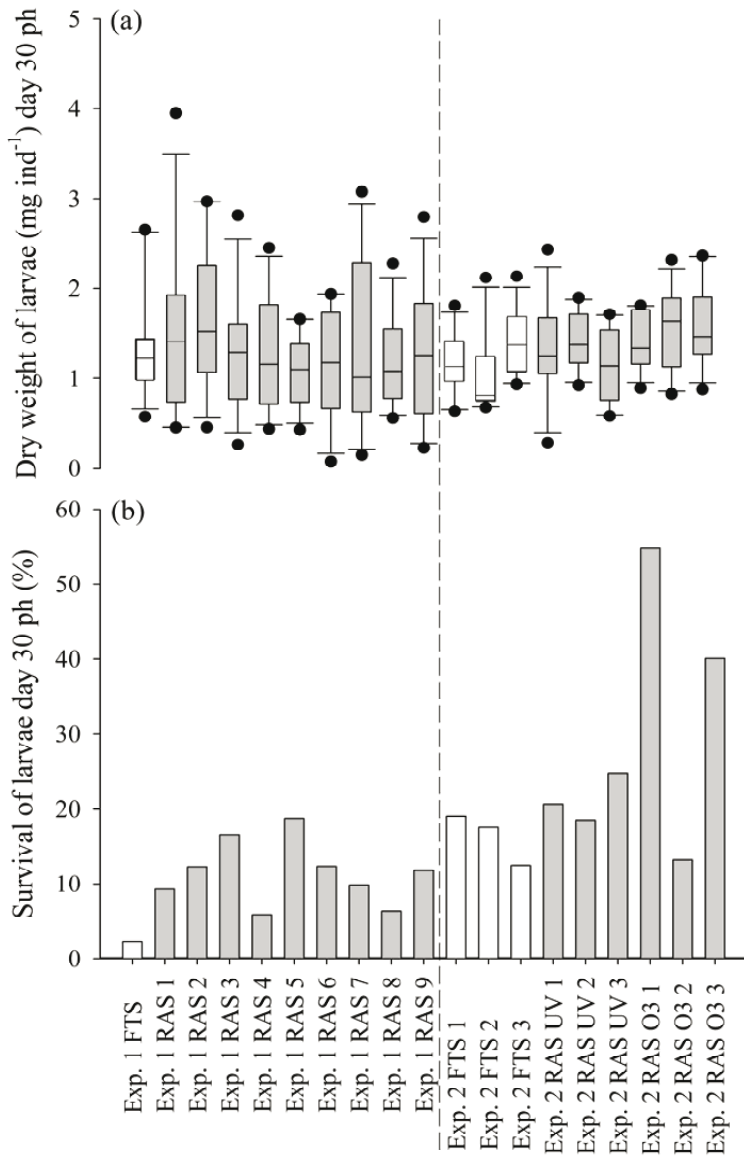
Fig. 4: (a) Dry weight and (b) survival of the cod larvae after 30 days in the replicate tanks of the FTS and the RAS with ozonation in Paper 1 (the bar for the FTS shows the average of 4 replicates) and in the FTS and the RAS with UV irradiation (RAS UV) and the RAS with ozonation (RAS O$_3$) in Paper 2. The boxes in plot (a) show the median and the 25th and 75th percentile, whereas whiskers indicate the 90th and 10th percentiles. Outlying points are marked (●).

Conclusions - Recirculation as a microbial control strategy
Selective enhancement of bacteria may be used to increase microbial stability and the fraction of neutral or beneficial bacteria at the expense of opportunistic and pathogenic types. Controlled selection is a matter of changing the possibilities in the stochastic variation of microbial colonisation. K-selection may be promoted to reduce the chances for opportunistic proliferation. K-strategists are favoured when the physiochemical environment and organic load is stable and the supply of substrate per bacteria is low. Consequently, the number of bacteria competing for substrate should be maintained close to a CC that is kept stable throughout the system. To maintain CC at a stable level, the load of organic matter may be reduced in the rearing tanks or increased in the intake water. Based on this rationale, the concept of RAS was suggested as a possible method for increasing microbial control. In a RAS the CC and the composition of the bacterial substrate is assumed to be relatively stable throughout the system. In addition the RAS is conservative and allow a long time and a large surface area of the biofilter for microbial maturation in the system. These properties were hypothesised to promote microbial stability and a more mature and beneficial bacterial community for larvae, which was supported by the results from two first feeding experiments. In both experiments the RAS with moderate ozonation generally developed and maintained a significantly different and more even and stable microbial community composition with a higher diversity of species and lower shares of opportunists compared to the FTS with the same feed and rearing regime. Atlantic cod larvae reared in the RAS performed equally well or better than their FTS siblings despite being exposed to an apparent inferior physiochemical water quality. The initial investigation suggests that RAS may be used as a strategy in the microbial management of marine hatcheries. However, further experiments are required to verify this, and to optimise design and operation for obtaining improved microbial water quality.

The accumulation of waste products is a drawback of RAS. Even if the level of waste was not considered to be problematic during the live feed period for the regime and systems tested, it may be suboptimal for the larvae. There is a potential for improving the physiochemical water quality in the hatchery RAS described in this thesis.

Future perspectives - Recirculation as a microbial control strategy
When the aim is to stabilise rearing conditions rather than saving water and energy, the level of water reuse may be adjusted to balance microbial stabilisation and physiochemical water quality. However, the level of water reuse needed to maintain a stable and beneficial microbial community is unknown. Algae or commercial fish feed may be used to increase CC in the heterotrophic maturing biofilter to compensate for a lower degree of reuse. The relative importance of the composition of the organic matter used to increase CC and the time to mature in the RAS on the improvement of the microbial composition is not known.

Separation of nitrification and heterotrophic maturation in different biofilters may increase efficiency by securing optimal selection pressure for each process. A design where fine solids are removed from a side stream of water going from the heterotrophic biofilter to the nitrifying biofilter could minimise organic load and competition for space and oxygen with heterotrophs in the nitrifying biofilter. If, for example, membrane filtration is used, the concentrated organic matter retained can be fed back to the heterotrophic filter for further degradation and production of bacteria.

Other strategies for microbial control may have the potential to work in synergy with RAS maturation, like the addition of probiotic bacteria to the rearing water. Probiotic bacteria can

be challenging to maintain in the system as they are not always favoured by the prevailing terms of selection. Some of the probiotic candidates may be selected on the basis that they are great colonisers of larvae, which is an r-strategic trait. If specific K-strategic beneficial bacteria are identified, the K-selective pressure thought to be created in the RAS combined with recycling of probionts may prolong their stay in the system compared to a FTS nursing opportunists and constantly diluting bacteria from the tanks. The addition of specific beneficial K-strategists may be a way to increase the control of the stochastic colonisation of the rearing tanks in a RAS.

5.3 The effects of hygienic barriers on the microbial environment in RAS for marine larvae

5.3.1 Biotic and abiotic particles protect marine heterotrophic bacteria during UV and ozone disinfection

The dose requirements for disinfection of seawater are deviating. In hatchery RAS particles and organic matter accumulates and turbidity is very high during the "green water" period, which may reduce disinfection efficiency for both UV-irradiation and ozonation. UV transmittance is reduced by turbidity and may be blocked by particles, whereas the oxidative power of ozone and rest oxidants can be reduced due to disinfectant degradation at the surface of suspended solids and the rate limited transport into particles (Perrins *et al*., 2006).

To study the effect of particles on the efficiency of disinfection free living bacteria and bacteria associated to live rotifers were exposed to UV-irradiation or ozonation. The bacteria associated with rotifers were protected from ozonation and showed a significantly higher survival than the free-living bacteria in all the ozone dose-response experiments (Figure 5). Water exposed to an average ozone dose of 49 mg s L^{-1} resulted in 99.9% inactivation of free-living bacteria but only 91.4% inactivation for particle (rotifer) associated bacteria. The trend was similar for the UV irradiation dose-response experiments, but the difference in inactivation of free-living compared to particle associated bacteria was smaller (Figure 6). A six fold increase in UV-disinfection dose (from 0.35 to 2.12 J cm^{-2}) was required to obtain 99.9% inactivation of the particle associated compared to the free-living bacteria.

UV-irradiated bacteria associated with rotifers experienced less protection when compared to bacteria in ozonated water. For both methods a two stage inactivation was observed, which is a deviation from first order kinetics. Inactivation of particle associated bacteria was less affected by an increased dose of ozonation than of UV irradiation, which may indicate a total residual oxidant (TRO) threshold level for the ozone dose. The dose response may be different at higher initial TRO concentrations. A higher survival of bacteria in the rotifers may be a result of passive protection or a sign of higher disinfection tolerance in the bacteria populating the digestive tract of the rotifer. However, strong correlations have been shown between the bacterial flora of the water and that associated with rotifers (Skjermo and Vadstein, 1993, Paper 3), making the presence of a more tolerant bacterial flora in the rotifers less likely.

Fig. 5: Survival versus dose relationship of free-living (open symbols) and particle associated (solid symbols) bacteria in ozonated rotifer cultures. Experiments Oz1 (◊, ♦), Oz2 (○,●) Oz4 (Δ, ▲) and Oz5 (□, ■).
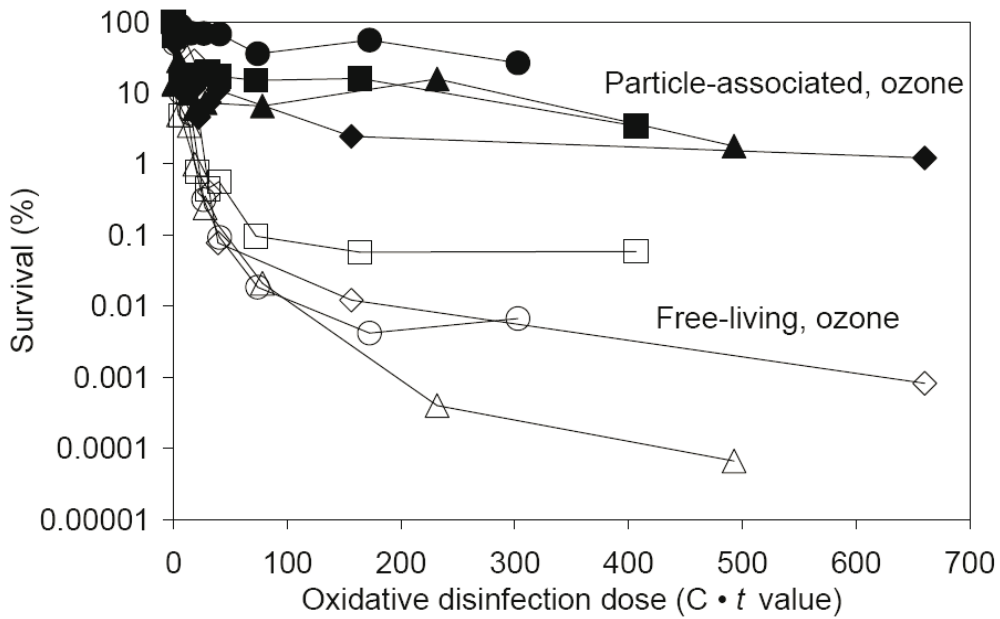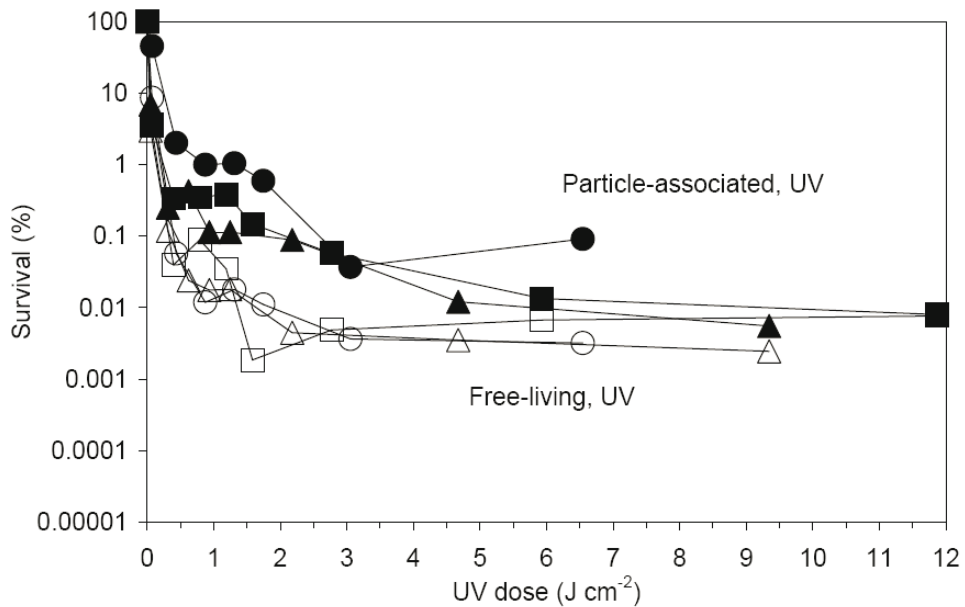

Fig. 6: Survival versus dose relationship of free-living (open symbols) and particle associated (solid symbols) bacteria in UV-irradiated rotifer cultures. Experiments UV3 (○,●) UV4 (Δ, ▲) and UV5 (□, ■).

The protection of free-living bacteria by increasing concentrations of biotic (rotifer) and abiotic (clay) particles was compared in a second set of experiments. An effect of particle concentration on survival was observed for both treatments, but with no clear differences between particle types (Figure 7). For the ozone treatment the survival of bacteria increased from 1.5% with no particles to 20% at particle concentrations above 65 and 85 mg dry weight L^{-1} for the abiotic and biotic particles, respectively. For the UV treatment the survival of bacteria increased from 0.03% with no particles to 0.1% at particle concentrations above 110 and 65 mg dry weight L^{-1} for the abiotic and biotic particles, respectively. This corresponds to a ~13 and ~3 fold increase in survival for the ozone and UV treatment, respectively.
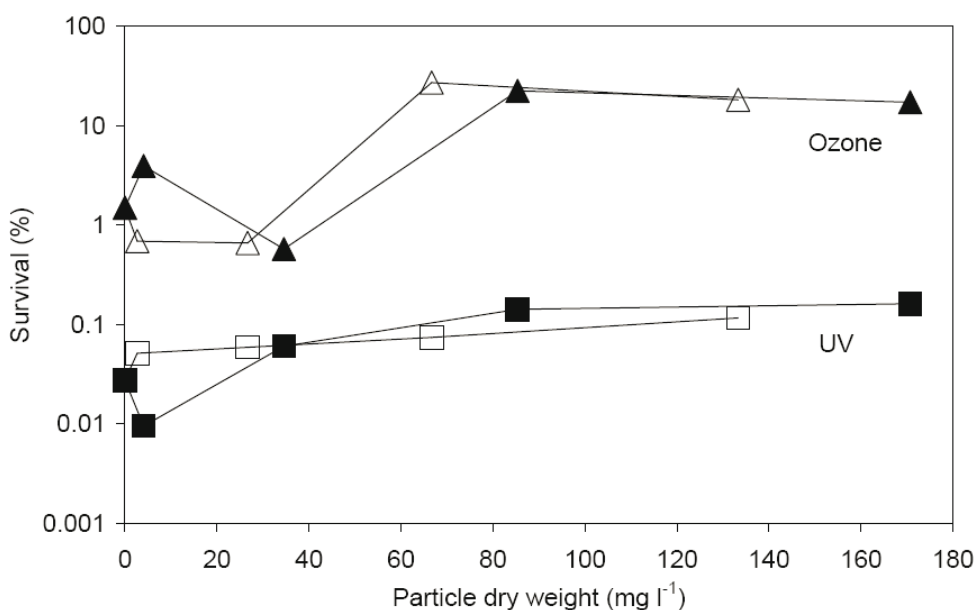
Fig. 7: Effect of biotic (rotifers, solid symbols) and abiotic (clay, open symbols) particle concentration on survival of bacteria after UV (□, ■) or ozone (Δ, ▲) treatment. The water was treated for 2 min, giving an average UV dose of 586 mJ cm^{-2} and an average C × t value of 30 mg s L^{-1}.

Protection of bacteria in the shadow of other particles seems likely for UV-irradiation (Liltved and Cripps, 1999), but should be absent with ozonation. Consumption of TRO or rate limited transport of disinfectants into aggregates may protect bacteria in ozonated water. However, the last is not likely for clay as bacteria are located only on the outer surface of the particle. The effect of particles on the protection of bacteria appeared to be stronger in ozonated than in UV-irradiated water. This may be explained by the consumption of TRO affecting the whole population of bacteria throughout the exposure, whereas shadowing from UV-irradiation is more limited in time because of the turbulence created by stirring in the reaction chamber. In conclusion, the two disinfection methods used showed different protection kinetics and mechanisms for heterotrophic bacteria.

5.3.2 The type of hygienic barrier and/or the disinfection efficiency used in RAS affect the development of the microbial community of the rearing water

UV-treatment and ozonation show reduced efficiencies of disinfection in seawater with a high concentration of particles (Paper 4), but may reduce bacteria numbers and influence the composition of the microbial community through selective inactivation, selective recolonisation and production (modification) of dissolved organic matter (Hess-Erga *et al.*, 2010).

RAS has been hypothesised to stabilise and mature the microbial community, creating a more beneficial environment for the larvae during the live feed period (Paper 1). The dose and mechanism of the hygienic measure most likely influences microbial selection, stabilization and maturation. This may influence the development of the microbial community in a RAS over time. Because RAS are commonly operated at low or moderate levels of ozonation, but with high intensity UV, the two methods are likely to represent different levels of disinfection efficiency. For each method, the intensity of disinfection is also likely to change through the rearing period, from low effect during the turbid "green water" period to higher inactivation of bacteria during the *Artemia* and dry feed period. It is possible that the order of water treatment in a RAS, i.e. disinfection before or after biofiltration, may also have effects on the development of the microbial community in the tanks.

In Paper 2 two RAS and a FTS were compared to study how the type and strength of a hygienic barrier influenced the development of the microbial community. The two RAS were identical apart from including either ozonation to 350 mV and two active carbon filters or UV-irradiation. Both the UV-irradiation and the ozonation happened before the water was biofiltered. The varying turbidity and fluctuating levels of particles and organic matter in the hatchery RAS made it difficult to determine specific disinfection doses of UV irradiation or ozonation during operation. However, an indication of the efficiency of the hygienic barrier was obtained by comparing the number and activity of bacteria in the treated water to that of the water in the tanks (Figure 8). The abundance and activity of the bacteria in the treated water in the RAS O_3 was similar to the water in the tanks (109 ± 12 % and 83 ± 1 % of the level in the incoming water, respectively), whereas the treated water in the RAS UV tended to have less bacteria and a lower activity than that of the rearing tanks to which it was distributed, indicating higher disinfection efficiency (38 ± 7 % and 43 ± 11 % of the level in the incoming water, respectively).

Both RAS stabilised the microbial community compared to the FTS (PCA of T-RFLP data, see *3.2 Recirculation as a microbial control strategy*), but there was a general trend of a more matured bacterial community in the RAS O_3 than in the RAS UV. The microbial community of the FTS and the RAS O_3 differed most of the three systems and the RAS UV resembled the FTS more than the RAS O_3 in microbial composition, the abundance of bacteria and the level of microbial activity (Table 3, Figure 8). The species richness of bacteria was higher in the two RAS than in the FTS during the *Artemia* period ($p = 0.024$, one-way ANOVA), and the cultivability of bacteria was higher in the FTS during the *Artemia* and dry feed period ($p = 0.017$). The RAS UV had higher proportions of fast-growing bacteria than the other systems during the *Artemia* period ($p = 0.008$). The larvae in the RAS O_3 showed the best performance of the three systems despite being exposed to an apparent inferior physiochemical water quality compared to the FTS. The larvae of the RAS UV showed a similar performance to the larvae of the FTS during the live feed period, but higher average larval dry weight and survival through the dry feed period (see *3.2 Recirculation as a microbial control strategy*).
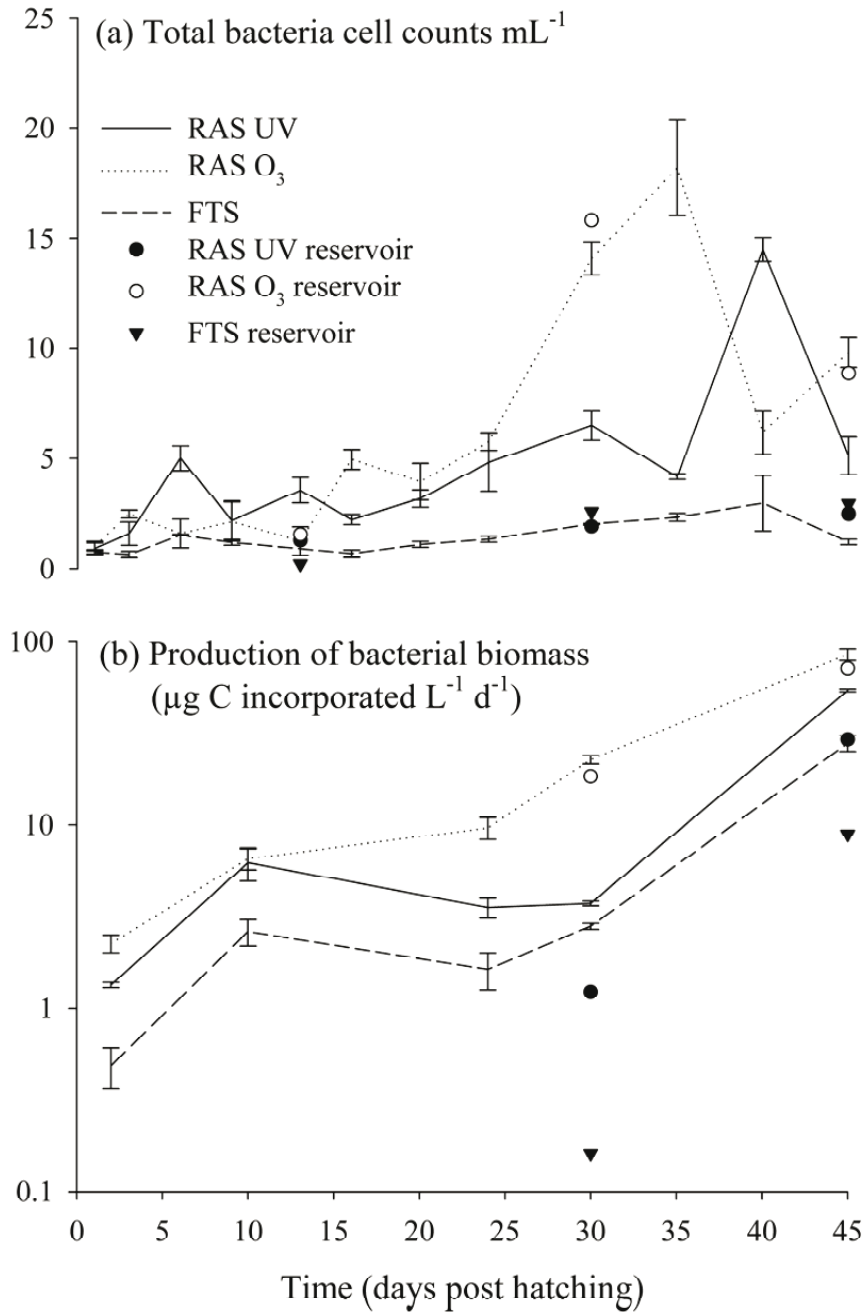
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Fig. 8: (a) Total bacteria cell counts and (b) the production of heterotrophic bacterial biomass (± SE) in the tanks and the water going to the tanks of the RAS UV, RAS O_3 and FTS. The production of heterotrophic bacterial biomass was estimated from the incorporation of ^3H leucine into macromolecules.

Two different processes can contribute to elevated stability of the microbial composition in RAS compared to FTS. First, RAS may passively conserve the microbial composition simply by retaining water in the system for a longer period of time, leaving less chance of random invasion. Secondly, RAS operation may exert a constant selection pressure on microbes, which results in a more stable microbial composition.

The results in Paper 2 indicated that the level or type of disinfection had effects on the ability of the RAS to mature the microbial community of the rearing water. We suggest that these results may be explained by the difference in disinfection efficiency between the UV treatment in the RAS UV and the moderate ozonation in the RAS O$_3$. Controlled selection of microbes in the water going to the tanks should theoretically be carried out at similar conditions to that found in the rearing tanks to maintain a stable ration of substrate per bacteria and to minimise the chance of proliferation of opportunistic species when the treated water enters the tanks. A reduction in the amount of bacteria competing for substrate may disturb the microbial community of the system, reduce maturity and create an opening for recolonisation and opportunistic proliferation (Salvesen *et al*., 1999, Paper 1).

The possibility that moderate ozonation in itself may have a beneficial effect on the microbial community development related to the fish or on the larvae directly cannot be ruled out. The difference in the level of maturation was small between the systems. Specific species of bacteria were not determined in this thesis, but the two RAS in Experiment 2 apparently included different species in the rearing water. Ozonation may contribute to or modify an environment that selects for beneficial bacteria or against some harmful types. Given the common empirical experience that a certain ORP level improves rearing conditions, it would be interesting to know whether ORP or TRO also influences microbial selection directly in fish tanks. It is also a possibility that moderate ozonation of the rearing water may directly affect the larvae. Little is known about the effect of artificial or natural ORP levels on microbe ecology or fish larvae in aquaculture or in the marine pelagic zone.

Conclusions - The effects of hygienic barriers on the microbial environment in RAS for marine larvae
Hygienic barriers like UV-irradiation or moderate ozonation are common in RAS. During the live feed period the turbidity and the concentration of suspended particles are high in marine hatchery RAS. Biotic and abiotic particles were shown to protect marine heterotrophic bacteria during UV and ozone disinfection. Generally, bacteria embedded in or attached to particles were better protected from inactivation than free-living bacteria. The protection of particle-associated compared to free-living bacteria was higher in ozonated water than in UV-irradiated water. The disinfection efficiency of free-living bacteria was reduced at higher concentrations of particles when the water was ozonated. For UV-irradiated water the negative correlation between inactivation of free-living bacteria and particle concentration was weaker.

UV-irradiation and ozonation have differing protection kinetics and inactivation mechanisms for heterotrophic bacteria. Most likely, the dose and mechanism of the hygienic measure may influence microbial selection, stabilization and maturation and the development of the microbial community in a RAS over time. Because RAS are commonly operated at low or moderate levels of ozonation, but with high intensity UV, the two methods are likely to represent different levels of disinfection efficiency. For each method, the intensity of disinfection is also likely to change through the rearing period, from low effect during the

"green water" period to higher inactivation of bacteria during the *Artemia* period. As expected, moderate ozonation (RAS O_3) did not reduce the number or activity of bacteria in the RAS, whereas UV-irradiation (RAS UV) showed disinfection.

RAS may be used as a means to increase and stabilise CC and mature the microbial community in the water going to the tanks. This involves the maintenance of a community close to CC with a stable and high number of bacteria competing for substrate. The type of hygienic barrier affected the development of the microbial communities in the two RAS in our study. Of the two, the bacterial community of the RAS O_3, which had the lowest disinfection efficiency, was characterised as more stable, mature and beneficial for the larvae. The RAS O_3 also generally had the best performing larvae of the experiment. The experimental results indicate that strong hygienic barriers may reduce the microbial maturation in the RAS. The reason may be that disinfection destabilises the microbial community by reducing the amount of bacteria competing for substrate.

On the other hand, little is known about the direct effects of ozonation of the rearing water on the microbial environment or the performance of marine larvae. It is possible that other effects than the low level of disinfection may have influenced the composition of bacteria in the rearing water. As an example, ozonation of seawater produces active compounds that remain in the system and may affect the selection of bacteria (Hess-Erga *et al*., 2010).

6 Recommendations

To control the development of the microbial environment in the fish tanks one has to control selection in the tank as well as the microbial composition and substrate supply coming from sources outside the tank. Based on the strategy presented by Vadstein *et al*. (1993, Figure 1, Table 1) and the results presented in this thesis, I suggest an approach to increase microbial control in the rearing of marine larvae which includes six focus points that are influencing the microbial environment experienced by the fish (Figure 9). The first four focus points are the elements that are added to the tank (eggs, water, particles and feed), whereas the two last points are the tank water and the fish larva itself. For each focus point I have formulated aims and suggested actions that may improve microbial control during the first feeding of marine larvae (Table 4).
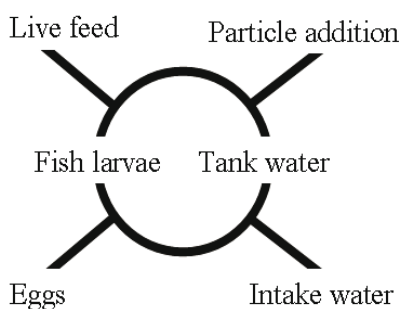

Fig. 9: Outline of six focus points that should be considered to be able to increase microbial control in the rearing of marine larvae.

For flow-through systems (FTS) it may be an advantage to combine microbial maturation of the intake water (after disinfection) with the addition of clay in the rearing tanks. Another strategy that was not investigated in this thesis, but should be looked in to, is feeding of the maturing unit of the intake water by a suitable substrate to increase CC to the level of the tanks. This could be a simple way to gain microbial control and increase stability in FTS for marine larvae.

For the live feed period in a marine RAS, moderate ozonation or no disinfection (Attramadal *et al.*, unpublished results) is suggested to promote the development of a more stable, mature and beneficial microbial community. When the aim is to stabilise rearing conditions rather than saving water and energy, the degree of water reuse may be balanced to optimise physiochemical water quality and microbial control. The degree of recirculation needed to maintain a stable and beneficial microbial community is not known. At some point down the production line, however, fish are more robust and addressing other challenges than microbial control become increasingly more important, i.e. oxygen demand and waste removal.

The effects of moderate ozonation (TRO or ORP) on the development of the microbial community should be investigated and the direct effects of moderate ozonation and the level and source of turbidity on the larvae should be evaluated to optimise the rearing regime.

In all the three first feeding experiments presented in this thesis the water treatment regime significantly affected the microbial environment and the performance of the fish, even if the rearing regimes used for control have been "best practise". In conclusion, the results of this thesis show that there is a potential for optimising the production of marine larvae by choosing the best water treatment for improving not only physiochemical conditions but also the larvae/microbe interactions. I recommend that further research should be carried out to secure and improve the physiochemical and microbial water quality of the rearing water with the overall goal to improve stability and quality in the production of juveniles of marine fish.

Table 4: An approach to increase microbial control in the rearing of marine larvae based on defined focus points (Figure 9).

Focus point	Aim	Action	References
Eggs	Minimise bacterial growth on eggs	Surface disinfection	Salvesen & Vadstein 1995; Salvesen *et al*., 1997
	Minimise transfer of harmful bacteria to tank	Microbial maturation of water	Skjermo *et al*., 1997; Salvesen *et al*., 1999
	Minimise transfer of organic matter to tank	High water exchange rates in incubator	
Intake water	Minimise transfer of harmful bacteria to tank	1. Disinfection, followed by	Salvesen *et al*., 1999, Paper 1
	Minimise the chance of proliferation	2. Regulation of microbial carrying capacity to resemble tank, followed by	
	of harmful bacteria in the tank	3. Microbial maturation	Skjermo *et al*., 1997
	Introduce neutral or beneficial bacteria	Addition of probiotic bacteria	Ringø & Vadstein, 1998; Makridis *et al*., 2000a
Particle addition	Minimise proliferation of harmful bacteria	Choosing the right species of algae for "green water"	Salvesen *et al*., 2000
		Choosing the right production regime for growing live algae	Salvesen *et al*., 2000
	Minimise transfer of organic matter to tank	Replacing algae with clay	Paper 3
		Choosing the right type of clay	
Live feed	Minimise transfer of harmful bacteria to tank	Cleaning outside of live feed	
		▶ Disinfection	Munro *et al*., 1995
		▶ Cleaning with intake- or tap water	
	Introduce neutral or beneficial bacteria	Replacing gut flora of live feed	
		▶ With microalgae	Olsen *et al*., 1999
		▶ By addition of probiotic bacteria	Makridis *et al*., 2000b
		▶ By microbial maturation of water	Skjermo *et al*., 1997
Fish larvae	Improve larval resistance against infections	Improvement of immune system	Skjermo *et al*., 1997, Vadstein, 1997
		Optimising and stabilising physiochemical water quality	
		Optimising welfare and minimising negative stress	
		Optimising nutrition	
Tank water	Minimise the proliferation of harmful bacteria	Continuous and efficient removal of waste	Paper 1 & 2
		Reuse of water or recirculation with low level/without disinfection	
		Optimising water exchange rates	
	Introduce neutral or beneficial bacteria	Addition of probiotic bacteria	Ringø & Vadstein, 1998; Makridis *et al*., 2000a

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Paper 1

Recirculation as a possible microbial control strategy in the production of marine larvae

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Abstract

Marine hatcheries represent high value, low waste systems for larvae that are sensitive to general infections by opportunistic bacteria. In intensive cultivation several procedures destabilise the microbial community of the rearing water and favour growth of potentially harmful microbes. Recirculation aquaculture systems (RAS) have properties that may contribute to microbial stabilisation, including long water retention time and a large surface area of biofilters for bacterial growth. Moreover, the microbial community in RAS biofilters has the possibility to stabilise at a similar carrying capacity as the larvae tanks, which could potentially limit the chances of proliferation of opportunistic microbes in the rearing water. The development of the microbial community in a RAS with moderate ozonation (to 350 mV) was compared to that of a conventional flow-through system (FTS) for the same group of Atlantic cod, *Gadus morhua*. The feed and intake water was the same for the two groups. The RAS developed and maintained a more diverse and stable microbial community composition compared to the FTS. Water treatment regime explained most of the variation in microbial composition during the live feed period, and in addition it accounted for ten times higher variation in the composition of bacteria in the FTS than in the RAS. Less variability in bacterial composition was found between replicate fish tanks of the RAS than between tanks of the FTS. The RAS had a more even microbial community structure with higher species diversity and periodically a lower fraction of opportunists. The fish in the RAS performed better than their siblings in the FTS, despite being exposed to an apparent inferior physiochemical water quality.

1 Introduction

Marine fry production of cold water species is steadily increasing, but challenges like suboptimal performance and poor reproducibility between replicate tanks are still typical in cultivation of early stages of cod, halibut, turbot, lobster and scallop (Olsen *et al*., 1999a, Verner-Jeffreys *et al.*, 2003, Jensen *et al*., 2004, Magnesen *et al.*, 2006). The poor reproducibility has been attributed to detrimental larvae-microbe interactions based on improvements obtained in studies using antibiotics (Munro *et al*., 1994, Skjermo *et al*., 1997, Verner-Jeffreys *et al.*, 2004) or microbial maturation of water (Skjermo *et al.*, 1997, Salvesen *et al*., 1999, Skjermo and Vadstein, 1999). Newly hatched marine fish larvae lack a fully functional specific immune system (Vadstein, 1997, Schrøder *et al.* 1998), and are vulnerable to general infections by opportunistic bacteria (Munro *et al*., 1994, Skjermo *et al.*, 1997, Hansen and Olafsen, 1999, Skjermo and Vadstein, 1999, Verner-Jeffreys et al., 2003, Sandlund *et al*., 2010). Clearly, there is a need for reliable and cost efficient microbial control strategies.

Vadstein *et a*l. (1993) used a number of criteria based on the ecological theory of r/K-selection (MacArthur and Wilson, 1967) to describe the state of microbial maturity of rearing water. Generally, a low maturity situation with a high share of fast-growing r-strategic bacteria is believed to be detrimental for fish larvae (Vadstein *et al.*, 1993). The carrying capacity (CC) is the number of bacteria that can be sustained in the system over time. Dissolved organic matter (DOM) is typically the growth limiting factor defining CC for heterotrophic bacteria, the group interacting directly with fish larvae. Microbially matured water contains a more diverse and resilient microbial community of K-selected specialists in abundance close to the carrying capacity (CC).

The microbial state of the rearing water depends on the supply of bacteria and organic matter together with selective forces in the tank and in the water sources. The main contributors of bacteria to marine hatchery tanks are live feed (Skjermo and Vadstein, 1993, Blancheton and Canaguier, 1995, Olsen *et al*., 1999b), algae (Salvesen *et al*., 2000) and intake water, as eggs are commonly surface sterilised (Salvesen and Vadstein, 1995). Live feeds are commonly associated with high levels of opportunistic bacteria (Skjermo and Vadstein 1993, Salvesen *et al.*, 1999, Olsen *et al.*, 2000).

In intensive larval rearing several procedures may destabilise the microbial community and favour proliferation of opportunistic and potentially harmful species. Traditional flow through systems (FTS) provide fish tanks with water containing relatively little organic matter and few bacteria. Disinfection of the intake water is a necessary precaution against pathogenic intruders, but at the same time the procedure temporarily diminishes competition between different types of bacteria. High densities of fish, algae and prey organisms cause increased and fluctuating organic load and hence a higher bacterial CC in the fish tanks. The enriched and perturbed environment with reduced competition promotes r-selection, i.e. opportunists (Hess-Erga et al., 2010). The problem is primarily not the level of organic matter per se, but the sudden change to higher concentrations that opens up for colonisation.

Microbial maturation of intake water is a well documented strategy that counteracts the destabilising forces through controlled microbial recolonisation, improving the performance of fish larvae the first days of rearing (Vadstein et al., 1993, Skjermo et al., 1997, Salvesen et al., 1999). A K-selective pressure is created by reducing nutrient supply per bacteria. In practice this means letting the biomass of the microbial community adjust to the CC post treatment by allowing some retention time in a reservoir with a large surface area occupied by K-strategists (Skjermo et al., 1997). However, because microbial maturation in FTS is related to the relatively low microbial CC of intake water, the transition to significantly higher substrate levels in the rearing tanks still represents a potential opening for opportunistic proliferation. Water with a bacterial community selected for at a CC closer to the levels in the enriched rearing tanks should hypothetically maintain higher microbial stability. Realizing this, Salvesen et al. (1999) encouraged the development of systems with capacity to produce microbially matured water at a CC matching that of the rearing tanks. Conceptually, a recirculating aquaculture system (RAS) may fulfil these criteria, and this type of system is therefore a possible candidate to mediate microbial control. If no strong disinfection barriers are applied, RAS allow a long water maturation time in the system. Analogous to a FTS maturation unit, a large surface area is available for growth of K-selected bacteria in the RAS biofilter. The heterotrophic population of the biofilter consumes organic matter and influences the microbial load and composition of the water proceeding to the fish tanks (Blancheton and Canaguier, 1995, Leonard et al., 2000, 2002, Michaud et al., 2006, 2009). Even when some of the particles are removed, RAS biofilters are supplied with water containing a DOM concentration and composition comparable to that of the water in the rearing tanks. These properties of the RAS may reduce the chances of detrimental opportunistic bacteria blooms in

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the rearing water, improving the microbial environment of the fish larvae. As far as we can see, this effect should theoretically be present in most RAS without being operated specifically for the purpose, but may depend on the efficiency of disinfection in the system (Attramadal *et al.*, submitted). Moderate ozonation (~350 mV) represent low level or no significant disinfection in a marine RAS (Hsieh *et al.*, 2002, Attramadal *et a*l., submitted). In the absence of a convincingly better physiochemical water quality, due to waste accumulation, a more stable and beneficial microbial environment was suggested to explain the improved performance of larvae obtained in two RAS compared to a FTS for Atlantic halibut, *Hippoglossus hippoglossus* (Verner-Jeffreys *et al.*, 2004), which is in accordance with our postulated hypothesis (Vadstein *et al.*, 1993, Salvesen *et al.*, 1999).

RAS are widely used to stabilise physiochemical water quality, reduce water consumption and control waste emission. We carried out an initial investigation of whether recirculation with moderate ozonation may be a possible mean for microbial management in production of marine larvae, by stabilisation and K-selection of bacteria. The development of the biomass and the composition of the microbial community in a RAS with moderate ozonation (to 350 mV) were compared to that of a conventional flow-through system (FTS) for the same group of Atlantic cod, *Gadus morhua*, from hatching until the introduction of formulated feed. The feed and intake water was the same for the two groups. The criteria used to define a better, more mature microbial water quality included: 1. A more persistent composition of the microbial community over time and less variability between parallel fish tanks; 2. A more K-dominated microbial flora with higher diversity and a lower fraction of general opportunists. In addition, the performance of the fish reared in the two systems was evaluated.

2 Materials and methods

2.1 Experimental setup

Figure 1 shows a flow scheme of the pilot scale RAS at Fosen Aquasenter AS, Norway. The systems were located in the production building of the commercial hatchery and the live feed was produced by the staff. The intake water (70 m depth) for the RAS and FTS was UV-filtered (BersonInLine 450, Berson UV-techniek, The Netherlands). Both systems included a cooling /heating system (E. Tydal AS, Norway). The FTS consisted of a 250 L reservoir with a vacuum pump (Rietschle VTE3, Gardner Denver Inc., USA), a pump (Flygt AP 26/2, ITT

Industries, Sweden) and four 250 L green coned circular polyethylene fish tanks. The RAS included nine 150 L black coned circular polyethylene fish tanks, a 500 L reservoir, a cylindrical 700 L biofilter filled with 350 L of water, and a 100 L protein skimmer (0.2 × 1.5 m) with a venturi pump feeding air and ozone from a generator (A1000, Erwin Sander Elektroapparatebau GmbH, Germany). The oxidation reduction potential (ORP) was measured continuously by a platinum electrode in the protein skimmer outlet and connected to a Redox-Potential Meter (Erwin Sander Elektroapparatebau GmbH, Germany) which secured 350 mV. Moderate ozonation to an ORP of ~350 mV in a marine RAS means very low disinfection efficiency, and is mainly included to improve general water quality and not to inactivate bacteria (Summerfelt, 2003, Tango and Gagnon, 2003, Attramadal *et al*., submitted a). In the biofilter a rotating perforated tube spread water over polystyrene beads (2-3 mm diameter beads, ~0.25 m^3 total substrate volume, 250 m^2 surface area). Strong aeration was secured from a bottom diffusor. The hydraulic retention time (HRT) of the biofilter was 0.06 h, receiving equal amounts of water from the reservoir and the protein skimmer (HRT 0.03 h). 5 g ammonium chloride (NH$_4$Cl) was added to the biofilter the day before transference of eggs. Tank outlets were central perforated pipes covered with nylon net (400 μm first 20 dph, then 800 μm) with air diffusors at 20 cm depth. Tanks were equipped with surface skimmers to collect wastes gathered in the surface film.
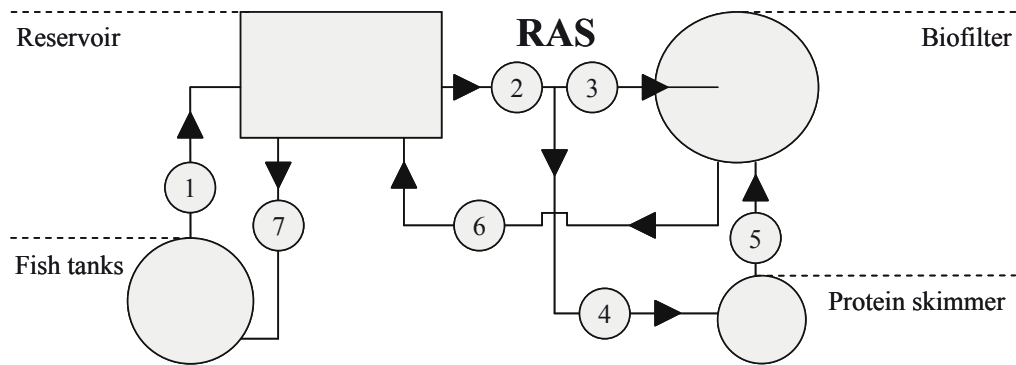
1 From fish tanks
2 From reservoir, 100 L min^{-1}
3 To biofilter, 50 L min^{-1}
4 To protein skimmer, 50 L min^{-1}

Fig. 1: Flow scheme of the RAS (not to scale).

2.2 Rearing regime

Atlantic cod, *Gadus morhua*, eggs (81 % fertilization success, as stated by the provider) were received from Troms Marin Yngel (TMY, Norway) at 49°d. The eggs were disinfected in 400 ppm glutaraldehyde for 6 minutes upon arrival (Salvesen and Vadstein, 1995), and incubated at 8°C in hatching incubators to about 73°d, when they were transferred to the experimental tanks at a final density of 150 individuals L^{-1}. The hatching success was 99 %. Larvae were hatched and maintained in darkness with low aeration the first 3 days, then exposed to continuous light and increased aeration (Table 1). The fish tanks of the two systems were in the same room and exposed to the same light. Aeration was stopped from day 22 ph, when the exchange rate of the tank water was high enough to provide sufficient circulation of water on its own. Water exchange rates were gradually increased from 1.5 to 10 tank volumes d^{-1}. 100 L of water was removed from the RAS during cleaning each day and replaced with intake water in the reservoir. Temperature was increased gradually from 8 to 12°C. *Nannochloropsis oculata* algae paste (Reed Mariculture, California) was distributed (1 mg C L^{-1} final concentration; Reitan *et al.*, 1993) to the fish tanks together with rotifers (*Brachionus plicatilis* Nevada) from day 2 to day 24 ph. The batch cultured rotifers were fed *N. oculata* algae paste, Baker's yeast and Marol-E (rotifer and *Artemia* oil emulsion enrichment, SINTEF, Norway). *Artemia* nauplii were hatched from INVE EG cysts with Hatch Control (INVE, USA) and short time enriched with Marol E before they were fed to the fish (day 19 to 31 ph). Live feed organisms were washed in sea water and concentrated before they were fed to the larval tanks three times a day to obtain a total tank concentration of about 3 individuals mL^{-1}.

Due to poor survival, the three FTS tanks containing the most fish (as judged by visual examination) were pooled in two tanks at day 30 ph. The pooling of FTS larvae was done by slowly reducing the water volume of the tanks and carefully collecting the larvae in buckets to be distributed evenly to two of the FTS tanks. The tanks were briefly emptied, but not cleaned before the larvae were moved. From day 30 ph the larvae were fed dry feed for two weeks. *Artemia* was co-fed as the dry feed was introduced the first two days. Three of the RAS tanks and one of the FTS tanks were fed Gemma Micro 300 (Skretting, Norway), whereas the other FTS tank received an experimental feed A. The remaining RAS tanks were fed an experimental feed B or C (three tanks each). The samples taken during the dry feed period are from the tanks receiving Gemma Micro 300. We are aware that the experimental feeds B and

C may have affected the selection of specific species of bacteria also in the three tanks fed Gemma Micro through the common water treatment system. However, the results of the microbial analysis showed that the microbial community composition of the two systems fed Gemma Micro resembled each other during this period (Figure 4), which indicated that the influence from the experimental feed types in the RAS was probably not very important for the development of the microbial community. The focus of our study is on the live feed period, but the dry feed period is included to get the microbial community development in a context and to see how the larvae coped with the challenge of accepting dry feed. Feeding dispensers (Fish Mate F14, Pet Mate ltd., UK) released dry feed once per hour. The ration of 1 g feed 100 L^{-1} water d^{-1} during the co-feeding period was gradually increased to 7 g 100 L^{-1} d^{-1}. Tanks were cleaned (careful siphoning of the bottom) every other day during the live feed period and every day during the dry feed period.

Table 1: Rearing regime.

Days post hatching	0	1	2	3	4	5	- 10	11	12	- 17	18	19	20	21	22	23	24	25	- 30	31	32	- 39	- 51				
Feed					Algae paste ⟶																						
					Rotifers ⟶																						
												Artemia ⟶															
																			Dry feed ⟶								
Water exchange (tank volume d^{-1})	1.5				2	4		5	6		7		8										10 ⟶				
Light	Darkness	Light ⟶																									
Temperature (°C)	8	9	10	11	12 ⟶																						
Aeration in tanks	Weak ⟶	Stronger ⟶												None ⟶													

2.3 Analytical procedures

Water quality and bacterial load and composition was measured or sampled before tanks were cleaned and fed in the rotifer period, but after the first feeding during the *Artemia* period, at random points in two fish tanks in each system. Water for particle counts, fixation for fluorescence microscopy, filtration for DNA extraction and agar plating was screened (50 μm) to exclude live feed organisms.

2.3.1 Performance of larvae

Larvae sampled for carbon biomass analysis (*n* = 12-20, depending on sample day) from one and the same tank in each system were sacrificed with an overdose of Tricaine Methanesulfonate (MS222), rinsed in fresh water and transferred to individual tin cups (Mikro Kemi AB, Sweden), dried (60ºC, 48 h) and analyzed in a CHN Elemental Analyser

1106 (Carlo Erba Instruments, Italy) at 1020ºC. Carbon and nitrogen contents were quantified chromatographically using standard curves obtained by analyzing acetanilide (C_6H_9NO). Individual dry weight (DW) of larvae was calculated from measured carbon content using a conversion factor of 2.34 (Reitan *et al*., 1993). Daily percentage specific growth rate (% SGR) was calculated from larval dry weight (DW) at time t according to Equation 1 and 2:

$$SGR \ (d^{-1}) = (\ln DW_t - \ln DW_0) \ / \ (t\text{-}t_0) \tag{1}$$

$$\% \ SGR \ (d^{-1}) = (e^{SGR-1}) \times 100 \ \% \tag{2}$$

High air and low water temperature increased RAS and decreased FTS temperatures, respectively, despite heating/cooling. This system difference in temperature (1°C) was corrected for by comparing larval size at the same day degree. This was calculated from individual dry weight and SGR for each sample time according to equation 3 and 4:

$$\text{Ln } DW_{corrFTS} = \ln DW_{ind} + SGR_{FTS} \times d°_{average}/ °C_{averageFTS} \tag{3}$$

$$\text{Ln } DW_{corrRAS} = \ln DW_{ind} - SGR_{RAS} \times d°_{average}/ °C_{averageRAS} \tag{4}$$

This correction allowed presentation and statistical comparison of larval dry weight at a common experimental period temperature average of 11.7 ºC.

Total numbers of surviving fish were determined at 45 dph after termination with an overdose of MS222. Survival at the end of the live feed period (30 dph) was calculated from daily mortality counts in the dry feed period (30 to 45 dph). Due to rapid degradation of dead larvae, the calculated numbers of survivors at day 30 ph represents a minimum. Larvae reared in the 4 FTS tanks during the live feed period were accidentally not counted before they were pooled and redistributed to two tanks, leaving no information about between-tank variation in survival at day 30 in the FTS.

2.3.2 Analysis of microbial communities

2.3.2.1 Colony forming units and fast-growing bacteria

The number of colony forming units (CFU) was determined from growth on M-65 seawater agar (Salvesen and Vadstein, 2000). Three 10-fold dilutions were plated from each sample, and each dilution was plated in triplicate. Samples were incubated in darkness at $12 \pm 1°C$. Total CFU were calculated as the average of colonies after 14 days of incubation. Plates containing 30-300 colonies were preferably counted. Because r-strategic opportunists are characterised by high maximum growth rates in contrast to K-strategic specialists, the fraction of fast-growing bacteria of total CFU was used as a measure of the relative presence of opportunistic bacteria (Skjermo *et al.*, 1997, Salvesen and Vadstein, 2000). In this paper, the term opportunistic bacteria is used to denote the CFU emerging the first two days of incubation on M65 agar, as described by Salvesen and Vadstein (2000).

2.3.2.2 Total bacteria cell counts and the fraction of cultivable bacteria

The total numbers of bacteria cells (total cell counts) were determined via fluorescence microscopy (Hobbie *et al.*, 1977). Samples were fixed with formaldehyde (0.7 % final concentration) and stored dark at 4ºC. Two mL of sample was diluted with 3 mL milli-Q water and vacuum filtrered onto black polycarbonate filters (0.22μm 25mm diameter, Poretics Corp., California) on supporting mixed cellulose ester membrane filters (45μm 25mm diameter, Whatman, UK). Three mL of DAPI (4.6-diamidino-2-phenylindole, 1 mg L^{-1} dH_2O) was added to stain the bacteria for 10 minutes (Porter and Feig, 1980). The dye was removed by filtration and the filters stored dark and dry. Stained bacteria were counted in a fluorescence microscope (Zeiss Axioplan 2, Germany) at 1250× magnification using UV excitation. A minimum of 250 individual bacteria, in at least 5 different random squares on the filter, was counted for each sample. The fraction of cultivable bacteria (CB) was calculated as the total CFU divided by total bacteria cell counts. The CB may be used as an indicator of the relative amount of specialists present (Skjermo *et al.*, 1997).

2.3.2.3 Bacteria DNA extraction

Sampled water (50 mL) was filtered through sterile 0.2 μm hollow fibre syringe filters (DynaGard, Microgon Inc., California) to retain bacteria. Filter futes were stored at -20ºC. Excess water was removed by gentle centrifugation. DNA was extracted using a DNeasy tissue kit from Qiagen following the Gram negative bacteria protocol. DNA concentration in

extracts was estimated by use of a Quant-iT PicoGreen dsDNA Quantitation kit (Molecular Probes, USA). Samples were excited at 480 nm and fluorescence emission intensity was measured at 520 nm in a Hitachi F-3000 fluorescence spectrophotometer (Japan). DNA extracts were stored at -20ºC. Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) was used to study diversity, changes and differences in the development of the combined cultivable and non-cultivable fraction of the microbial community (Muyzer *et al.*, 1993, Liu *et al*., 1997, Clement *et al*., 1998).

2.3.2.4 Polymerase chain reaction (PCR)

The target region of 16S rDNA was amplified in a Hybaid Omnigene Thermal Cycler (USA) using a Qiagen *Taq*PCR Master Mix Kit, primers (0.4 µM) and extracted DNA (~250 ng mL^{-1} final concentration). Hot start (incubation of sample at 95ºC for 5 min and cooled to 80ºC before addition of polymerase) was used to minimize non-specific annealing of primers. The forward primer 8f (5' AGA GTT TGA TC(AC) TGG CTC AG; Giovannoni *et al.*, 1990) and the reverse primer 517r (5' ATT ACC GCG GCT GCT GG; Lane *et al*., 1985, Muyzer *et al*. 1993) was used (Sandaa *et al.*, 2003). A GC-clamp (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G; Muyzer *et al*. 1993) was attached at the 5' end of the forward primer when samples were prepared for DGGE. When samples were amplified for TRFLP, the forward primer was labelled with a 6-FAM (6-carboxyfluorescein) fluorescent tag at the 5' end. Table 2 shows the amplification protocols used. Negative controls were included in the runs. Amplification products were stored at -20ºC.

Table 2: Amplification protocols.

Step	Temperature (°C)	Time (minutes)	Number of cycles
For TRFLP			
Denaturation	95	5	1
Denaturation	94	1	
Annealing	55	1	25
Extension	72.5	1	
Extension	72.5	8	1
Deactivation	97	5	1
For DGGE			
Denaturation	95	5	
Annealing	60	1	1
Extension	72.5	1	
Denaturation	94	1	
Annealing	58	1	1
Extension	72.5	1	
Denaturation	94	1	
Annealing	56	1	1
Extension	72.5	1	
Denaturation	94	1	
Annealing	55	1	25
Extension	72.5	1	
Extension	72.5	8	1

2.3.2.5 Denaturing gradient gel electrophoresis (DGGE)

Casting and running of denaturing 8 % (w/v) polyacrylamide gels in 0.5×TAE buffer (20 mM Tris base, 10 mM acetate and 0.5 mM EDTA [pH 7.4]) were performed as described by Schäfer and Muyzer (2001) by use of a Höefer Scientific SE 600 Vertical Electrophoresis System (Höfer Inc., USA). The linear gradient of urea and formamide ranged from 30 % to 55 % denaturant, 100 % denaturant corresponding to 6.5 % acrylamide, 40 % formamide and 7M urea in 1×TAE buffer. 15 µl PCR product mixed with 3 µl loading dye buffer were loaded to each of 20 wells on two gels. Gel A contained samples from each system at frequent intervals from day 4 ph to day 45 ph, and gel B samples from parallel tanks from each system. Some samples were loaded to both gels to allow comparison of the gels. Electrophoresis was run at a constant voltage (60V) at 60°C for 20 h in 0.5×TAE buffer, and poststained for 1.5 h with SYBR green II (1:10000 dilution in 0.5×TAE, 20 mL on each gel, Molecular Probes, USA). The gels were photographed with a Polaroid camera with a filter for SYBR green (665 positive/negative film, Polaroid, USA).

2.3.2.6 Terminal restriction fragment length polymorphism (TRFLP)

PCR products were cut with the restriction enzyme *Hha* I (GE Healthcare, UK) at 37°C over night. The 30 µl cutting mixture contained PCR product (5 µl), 10×buffer (3 µl), *Hha* I (1 µl) and dH$_2$O. Digested PCR products were precipitated with 96 % ethanol and 3M NaAc, and the resulting DNA pellets were washed with 70 % ethanol, air dried at 37°C and stored at -20°C. The digested PCR products were added 10 µl denaturing solution (formamide and standard), before lengths of TRFs were determined in an ABI Prism Genetic Analyzer 3100 (Applied Biosystems, California) with 20 seconds injection time at 60 KV voltage. Resulting peak area data were standardized by the variable percentage threshold method (Osborne *et al*., 2006). The divisor that resulted in the weakest relationship between number of peaks remaining and initial total area was used for further calculations. Each TRF was considered an operational taxonomic unit (OTU) within the bacterial community (Engebretson and Moyer, 2003).

2.3.2.7 Analysis of DGGE and TRFLP data

Schematic drawings, binary data and cluster dendrograms of DGGE band patterns were obtained using the GEL2k software program (Svein Norland, Dept. of Biology, University of Bergen, Norway). Clustering of profiles was based on the simple matching algorithm, and dendrograms were drawn using the complete link method.

The range-weighted richness index (Rr) derived from the DGGE patterns were calculated for each sample (Marzorati *et al*., 2008). Shannon's diversity index (H') was calculated for the TRFLP profile of each sample, using OTU abundance for species richness. The interpretation of the abundance of DGGE bands and TRFLP peaks as species richness is a simplification that may not be valid for the actual population, as the total abundance of species is unknown (the number of species that is not represented in an amount that is large enough to pass the threshold of the method is unknown), but was used to give some idea of the relative distribution and evenness of the dominating species of bacteria.

Time based population changes based on the Bray-Curtis dissimilarity coefficient and the principle of moving window analysis (Possemiers *et al*., 2004) were calculated from data obtained from DGGE and TRFLP. The Bray-Curtis dissimilarity coefficient was also used to compare samples from replicate tanks within each system, and to describe the difference between samples from the two systems each sample day.

2.3.3 Physiochemical water quality

Water temperature and oxygen concentration was measured daily with a portable electrode (Handy Gamma, Oxyguard, Denmark). The pH was measured daily with a pocket pH meter (330i, WTW, Germany). The oxidation reduction potential (ORP) was measured by platinum redox electrodes (Erwin Sander Elektroapparatebau GmbH, Germany). Nitrogenous waste products (total ammonia nitrogen, TAN, nitrite nitrogen, NO_2-N, and nitrate nitrogen, NO_3-N, concentrations) were measured with a DR/890 HACH Colorimeter (HACH, USA). Unionized ammonia (NH_3-N) concentration was calculated from TAN and pH considering temperature and salinity. NO_3-N measurements was calibrated (standard addition curves) to the prevailing chloride concentration. Nitrate concentration of intake water was below detection limit (<0.8 mg L^{-1} NO_3-N). Dissolved organic carbon (DOC) samples were immediately vacuum filtered through ignited (480ºC, 2 h) 0.7μm, 25mm diameter GF/F glass microfiber filters (Whatman International Ltd., England). Filtrate was stored at -20ºC and analysed in a Tekmar-Dohrmann Apollo 9000 TOC-analysator (Teledyne Tekmar, USA). Particle samples (50 ml) were fixed with 1 ml acidified Lugol's solution and stored dark at 12ºC before they were analysed in a CASY 1 Cell Counter and Analyzer System, model TTC (Schärfe System Gmbh, Germany) with a 150 μm capillary. Lower measuring limit was 3 μm. Inversely filtered (0.2 μm) seawater was used as background.

2.3.4 Statistical analysis

Mean ± standard error of the mean (SE) or standard deviation (SD) is presented. Statistical analysis was performed at the 95 % confidence level ($p < 0.05$). Data for larval dry weight were log_{10} transformed to secure a variance independent from the mean, and tested for differences by t-tests together with the Levene's test for equality of variances in SPSS16.0 (SPSS Inc., USA). For % SGR, SE was calculated from linear regressions of log transformed individual DW data (Sigmaplot, Systat Software Inc., USA). The Levene's test for equality of variances and t-tests or Mann-Whitney tests were used to compare the amount of variation and significant differences, respectively, in water quality variables between the systems. Principal component analysis (PCA) of TRFLP fingerprints was performed using the PLS toolbox v5.2 from Eigenvector research under Matlab v7.2. PCA is an unsupervised method in which high numbers of possibly correlated variables are reduced into a smaller number of uncorrelated variables called principal components (PCs). The first principal component (PC1) accounts for as much of the variability in the data as possible, and each succeeding

component accounts for as much of the remaining variability as possible. The PCs are used to describe the degree of similarity between the samples. The standard deviation (SD) of the PC1 and PC2 score values was used as a measure of change in the TRFLP fingerprint. The $SD_{PC1} \times SD_{PC2}$ value is directly proportional with the amount of change observed. PCA results from the two treatments were compared in a Mann-Whitney U-test.

3 Results

3.1 Physiochemical water quality

There were no differences in the variation of DOC, temperature, oxygen concentration or pH over time between the systems (p = 0.074, 0.440, 0.519 and 0.110, respectively). Temperature was on average 12.3 and 11.2 ºC in the RAS and FTS, respectively (p < 0.001), and salinity was 34 ppt in both systems. Oxygen concentration never fell below 80 % saturation for any of the treatments, and the concentration of oxygen did not differ between the systems (p = 0.775). The pH declined in the RAS from the *Artemia* period and in the FTS in the dry feed period to reach values of 7.4 and 7.6 on day 40 ph, respectively, and was lower in the RAS than in the FTS on average during the experiment (p = 0.002). Intake water ORP was 195 mV. The variation of the ORP was higher in the RAS (p = 0.001). FTS tanks had an ORP fluctuating around 180 mV the entire experiment, with a slow decline (slope of -0.3 for 0-40 dph, r^2 = 0.09, Sigmaplot). The RAS had higher ORP than the FTS in the rotifer period, but ORP decreased faster in the RAS tanks (slope of -1.4, r^2 = 0.51), and was lower than in the FTS in the dry feed period. For the experiment as a whole, there was no significant difference in the level of the ORP between the systems (p = 0.727). The concentration of DOC was about twice as high in the RAS as in the FTS (p = 0.004). The particle counts were higher (p = 0.004) and more variable (p = 0.003) in the RAS, but within the same order of magnitude as in the FTS. Most particles were less than 4 μm in the live feed period, corresponding to the cell size of *Nannochloropsis oculata* (2-3 μm, Andersen *et al*., 1998). There was a peak in the number of 6 μm particles in the dry feed period.

Table 3: Physiochemical water quality in the RAS and FTS during the different stages of feeding (average ± SE).

Period	Rotifer (2-18)		*Artemia* (19-30)		Dry feed (31-45)	
System	RAS	FTS	RAS	FTS	RAS	FTS
Temperature (°C)	11.9 ± 0.2	10.5 ± 0.1	12.3 ± 0.2	11.7 ± 0.2	12.6 ± 0.2	11.8 ± 0.2
Oxygen saturation (%)	88 ± 1	89 ± 1	95 ± 2	98 ± 1	96 ± 1	96 ± 1
pH	8.0 ± 0.0	8.0 ± 0.0	7.9 ± 0.0	8.0 ± 0.0	7.5 ± 0.1	7.8 ± 0.1
Oxidation reduction potential (mV)	200 ± 8	180 ± 4	195 ± 6	190 ± 4	162 ± 4	175 ± 2
Total ammonia (μg TAN L^{-1})	147 ± 86	33 ± 27	28 ± 28	15 ± 15	104 ± 70	0 ± 0
Unionized ammonia (μg NH$_3$-N L^{-1})	2.2 ± 1.3	0.5 ± 0.4	0.2 ± 0.2	0.3 ± 0.3	0.6 ± 0.3	0.0 ± 0.0
Nitrite (μg NO$_2$-N L^{-1})	8.8 ± 3.6	0.0 ± 0.0	6.2 ± 0.5	0.0 ± 0.0	17.1 ± 4.0	0.1 ± 0.1
Nitrate (mg NO$_3$-N L^{-1})	2.6 ± 0.6	0.5 ± 0.3	3.2 ± 0.1	0.4 ± 0.4	6.7 ± 0.8	0.5 ± 0.3
Dissolved organic matter (mg DOC L^{-1})	2.1 ± 0.5	1.4 ± 0.2	1.9 ± 0.0	1.0 ± 0.1	2.7 ± 0.2	1.4 ± 0.4
Particles (sized 3-10 μm) counts μL^{-1}	8.3 ± 2.2	3.3 ± 1.1	5.1 ± 1.4	0.9 ± 0.1	2.8 ± 0.9	2.2 ± 0.5

The concentration of nitrogenous waste was higher ($p < 0.037$) and more variable ($p < 0.010$) in the RAS than in the FTS. There was a peak in the concentration of ammonia and nitrite in both systems during the first week post hatching. The starting point concentration of ammonia was different in the two systems, reflecting the addition of ammonia to the biofilter prior to transfer of eggs. Surplus ammonia from the biofilter increased the initial level of TAN in the RAS compared to the FTS by 0.14 mg TAN L^{-1} or 2 μg NH$_3$-N L^{-1}. As a consequence, larvae in the RAS experienced a higher peak of ammonia and nitrite during the first week than can be accounted for by their own production of nitrogenous waste. Nitrate accumulated gradually from the second week ph in the RAS and reached a maximum level of 8 mg NO$_3$-N L^{-1} at the end of the experiment.

3.2 Microbial environment

The abundance of bacteria was higher in the RAS than in the FTS ($p = 0.007$ for CFU and $p = 0.008$ for total cell counts, Figure 2 a and b). Both systems showed a bloom of bacteria during the first week post hatching, reaching levels of about 2000 times higher CFU and 50 times higher total counts than before eggs were added. The number of bacteria declined in the FTS during the *Artemia* and dry feed periods. The abundance of bacteria varied more in the RAS than in the FTS over time ($p = 0.001$ for CFU and $p = 0.020$ for total cell counts from 10 dph).
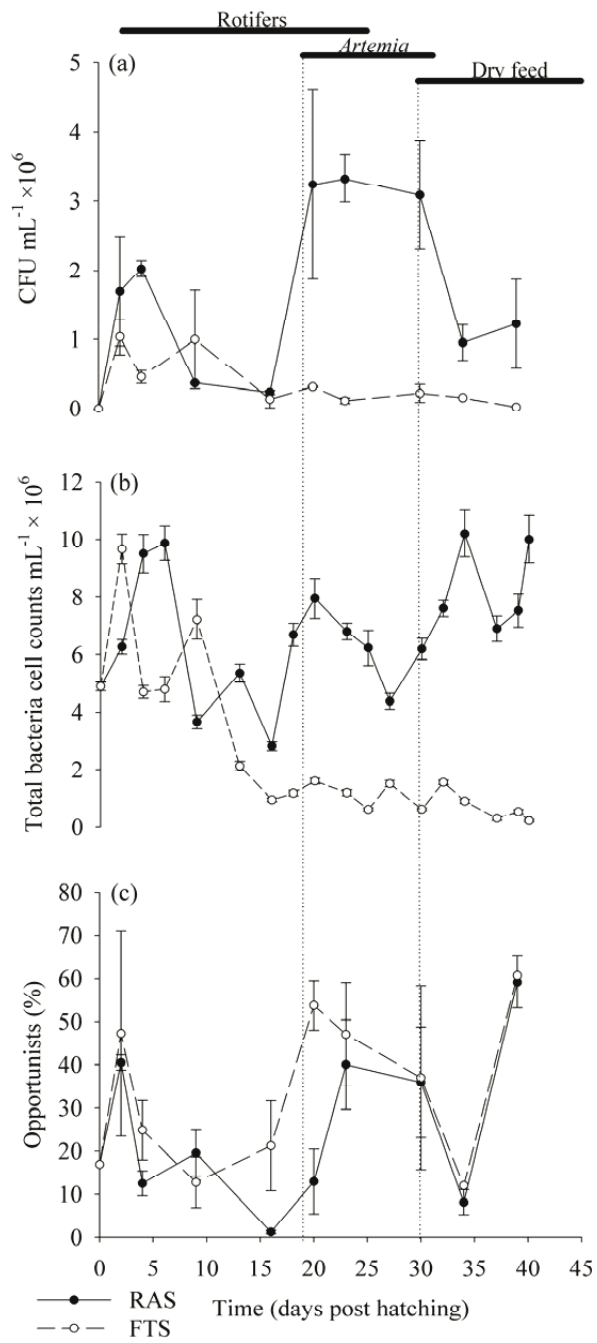
Fig. 2: (a) Number of colony forming units, (b) total bacteria cell counts, (c) the fraction of presumptive opportunistic bacteria (± SE) in the RAS (filled symbols) and FTS (open symbols).

Table 4: Characteristics of the microbial community in the rearing water of RAS and FTS (average ± SE). The total bacteria cell counts done via microscopy. The fast-growing opportunists are shown as relative fractions of total CFU. Cultivability is the proportion of CFU the total counts of bacteria. The time based population changes based on the Bray-Curtis dissimilarity coefficient and the principle of m... window analysis were calculated from the data obtained from DGGE and TRFLP. The Bray-Curtis dissimilarity coefficient was also use... compare replicate tanks in each system.

Period (dph)	Rotifer (2-18)		Artemia (19-30)		Dry feed (31-45)		Total (2-45)	
System	RAS	FTS	RAS	FTS	RAS	FTS	RAS	FTS
Direct counts of bacteria × 10^6 mL^{-1}	6.3 ± 1.0	4.4 ± 1.2	6.3 ± 0.6	1.1 ± 0.2	8.1 ± 0.7	0.8 ± 0.2	6.9 ± 0.5	2.3 ± 0.7
Colony forming units ×10^6 mL^{-1}	1.1 ± 0.5	0.7 ± 0.2	3.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.1	0.1 ± 0.1	1.6 ± 0.4	0.4 ± 0.1
Opportunists (%)	18 ± 8	27 ± 7	24 ± 8	45 ± 5	34 ± 26	36 ± 24	24 ± 6	34 ± 6
Cultivability (%)	17 ± 4	12 ± 1	47 ± 3	22 ± 7	13 ± 3	11 ± 6	26 ± 6	15 ± 3
Shannon's diversity index	1.7 ± 0.1	1.2 ± 0.4	1.9 ± 0.2	1.2 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.3 ± 0.2
Range-weighted richness index	13 ± 2	9 ± 2	13 ± 1	5 ± 1	11 ± 1	9 ± 3	13 ± 1	7 ± 1
DGGE band abundance	8 ± 1	7 ± 1	8 ± 0	6 ± 1	8 ± 1	7 ± 2	8 ± 1	7 ± 1
TRFLP peak abundance	9 ± 1	6 ± 2	14 ± 4	6 ± 1	12 ± 0	7 ± 1	12 ± 2	6 ± 1
Time based population changes % (DGGE)	14 ± 4	23 ± 6	14 ± 4	19 ± 10	12 ± 6	38 ± 0	18 ± 2	26 ± 5
Time based population changes % (TRFLP)	29 ± 5	48 ± 12	44 ± 8	69 ± 15	38 ± 5	83 ± 17	36 ± 5	63 ± 9
Bray-Curtis dissimilarity coefficient % of replicate tanks (DGGE)	0 ± 0	25 ± 5	3 ± 3	8 ± 8				

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The fraction of opportunistic bacteria was significantly higher in the FTS than in the RAS at day 4, 16 and 20 ph (p = 0.050, Figure 2 c). Variation between tanks was higher in the FTS for total CFU and presumptive opportunists. Cultivability varied more in the RAS than in the FTS (p = 0.030, Table 4), and was significantly higher in the RAS during the *Artemia* period (p = 0.035). Cultivability was twice as high in the *Artemia* period than in the periods with rotifers or dry feed in both systems.

Intake water had 2×10^3 CFU mL^{-1} and 5×10^6 total cell counts mL^{-1}. About 30 mL water from hatching incubators were added per fish tank litre together with the eggs during transfer. Hatching water contained 5×10^5 CFU and 1×10^6 total cell counts mL^{-1}. Even if live feed organisms were concentrated, a significant amount of washing water (~ 1 L) was transferred to fish tanks during feeding. Water associated with *Artemia* had 3×10^6 CFU mL^{-1}.

The range-weighted richness is reflecting the carrying capacity of the system. A higher Rr is characteristic of communities with a high microbial diversity, whereas the low Rr that was characteristic of the FTS (Rr < 10) is attributed to environments particularly adverse or restricted to colonization (Marzorati *et al.*, 2008). The diversity of the dominant species of bacteria, i.e. those that are detectable over the threshold of the PCR-based methods, was higher and more stable in the RAS (Abundance of DGGE bands and TRFLP peaks, Shannon's diversity index, range-weighted richness, Table 4). Population composition changed more in the FTS over the course of the experiment (Bray-Curtis dissimilarity index and moving window analysis of TRFLP and DGGE profiles, Table 4). The change of species composition was always higher after introduction to a new feed type and the number of DGGE bands and TRFLP peaks increased from start to end in both systems.

According to principal component analysis (PCA) of TRFLP data, PC1 and PC2 accounted for 61.3 % (44.6 % and 16.6 % for PC1 and PC2, respectively) of the variation in the data set. The SD$_{PC1}$×SD$_{PC2}$ value is directly proportional with the amount of change observed in the TRFLP fingerprints, and hence the amount of variation in the qualitative microbial flora in the systems. Consistent with the other analyses, the RAS showed a more stable bacterial flora with an SD$_{PC1}$×SD$_{PC2}$ of 0.02 compared to 0.05 for the FTS.

Two DGGE gels were run: gel A reflected the development of the microbial community in the systems over time, whereas gel B compared parallel tanks. Similar results were obtained

for identical samples loaded on both gels. There were apparent differences in species composition between the two systems. Figure 3 shows cluster dendrograms from the DGGE profiles on the two gels separating RAS and FTS samples. Through the experiment there was on average 59 ± 5 % dissimilarity between the microbial composition of the systems (Bray-Curtis dissimilarity index of TRFLP and DGGE profiles).

To study the variation of the microbial composition between the different feed type periods, the arithmetic mean of the PCA score values was calculated for each period and system. Figure 4 shows the trajectories of the two systems. Water treatment separated the sample scores on the PC1 axis, whereas feed type or time separated the scores along the PC2 axis. This is consistent with cluster analysis of DGGE data (Figure 3) and dissimilarity analysis (Table 2) showing that microbial composition in samples from one feed period was more similar compared to that in samples from the other feed periods. According to PCA analysis, the microbial composition of the two systems was significantly different in all feed periods for PC1 ($p = 0.006$, Mann-Whitney U-test), which accounted for the major part of the variation in the TRFLP profiles. The largest difference found in microbial community composition and development was thus related to the water treatment regime. The higher variation in microbial community composition in the FTS was also mainly connected to water treatment regime as the overall SD for the FTS along PC1 is the reason for its higher $SD_{PC1} \times SD_{PC2}$. Along PC2 there is a net increase in score values in both groups, with low SD_{PC2} values within each period compared to SD_{PC1} (except the rotifer period, RAS). Based on the two PCs of the PCA considered here, there was a general difference in microbial composition between the systems when all periods were seen together along PC1 and a similar development over time or with change of feed type seen along PC2. The systems had a more similar microbial composition in the earliest samples (9 and 13 dph). This was evident both from DGGE (Figure 3) and TRFLP data (data not shown).
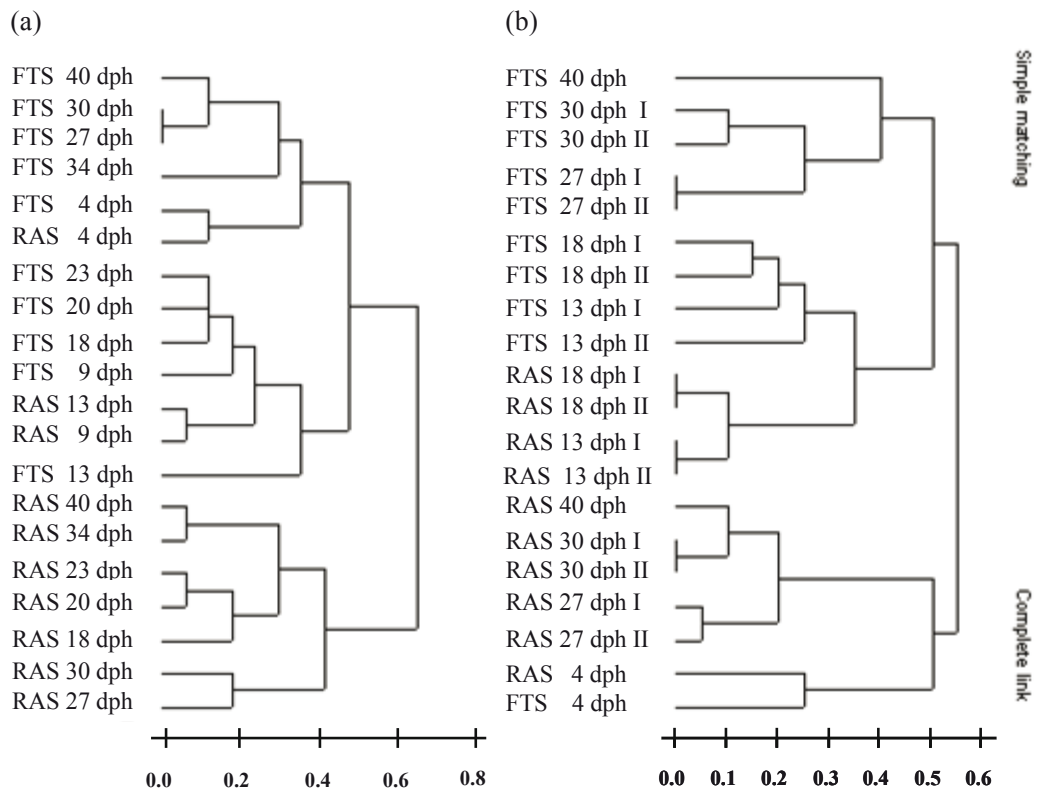
(a)

(b)

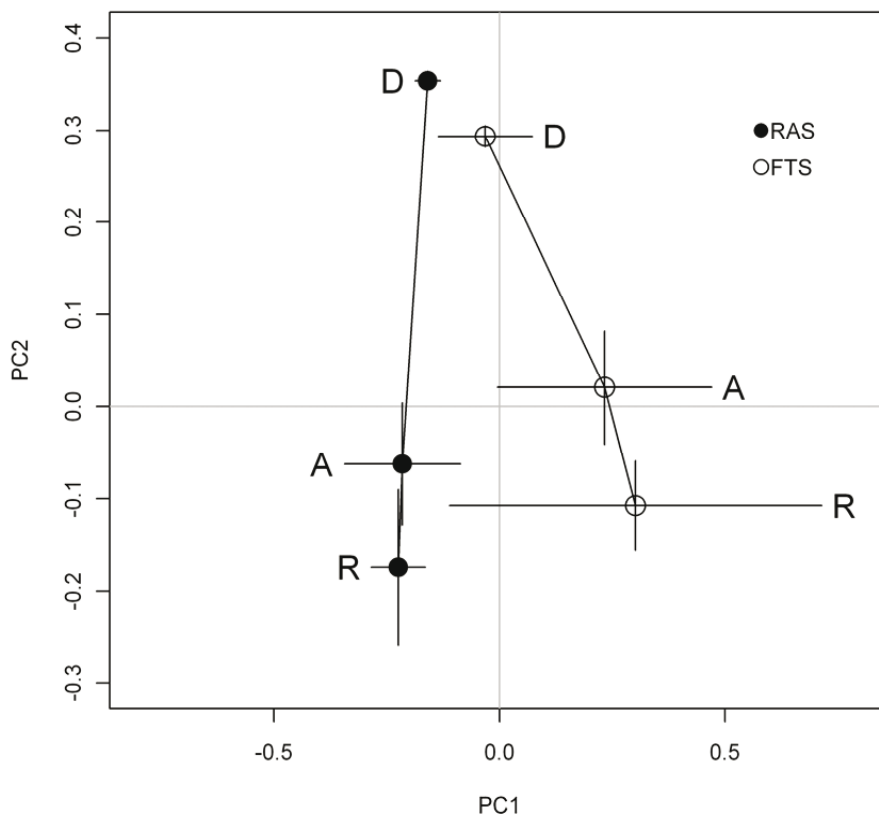**Fig. 3:** Cluster diagrams from DGGE (a) gel A and (b) gel B.

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Fig. 4: PCA score plot of TRFLP data with $SD_{PC1} \times SD_{PC2}$ and trajectory lines (R = rotifer period, A = *Artemia* period, D = dry feed period). The $SD_{PC1} \times SD_{PC2}$ value is directly proportional with the amount of change observed in the TRFLP fingerprints.

3.3 Fish performance

Figure 5 shows the gain in dry weight of the cod larvae and Table 5 shows a summary of the performance of fish in the two systems. The larvae lost some weight consuming yolk sac reserves. However, while the FTS larvae maintained a negative growth rate to day 5 ph, the RAS larvae had already started to put on weight at this point. The earlier net growth in the RAS was reflected in significant higher larval dry weight on day 5 ($p = 0.002$, Kruskal-Wallis). Most of the variability in final larval dry weight and density between parallel RAS tanks could be explained by density differences ($r^2 = 0.91$, regression). RAS larvae showed significantly higher survival through the dry feed period than FTS larvae ($P < 0.001$, Wilcoxon (Gehan) Statistics, life history analysis of survival).
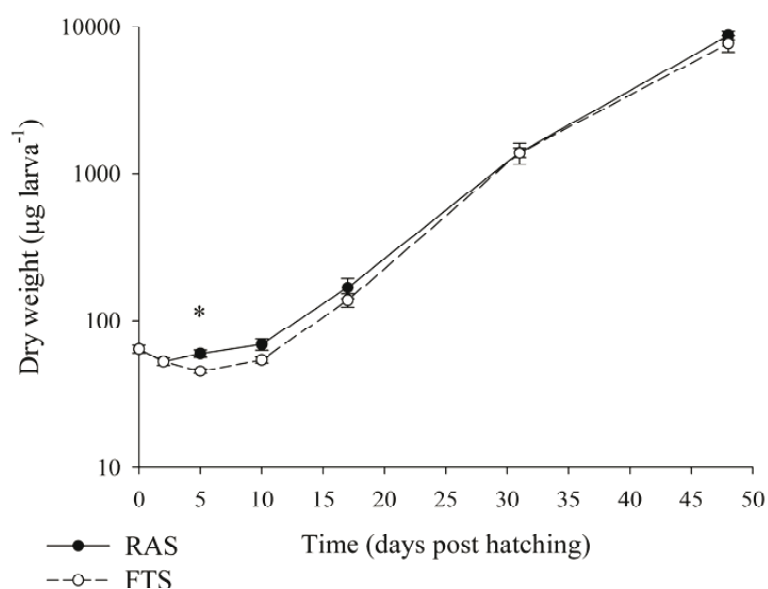
Fig. 5: Dry weight at an average of 11.7 ºC through the experiment (corrected for temperature differences, see *2.3.1 Performance of larvae*), asterisk marks significant difference between groups.

Table 5: Survival and growth of fish in the RAS and FTS (average ± SE).

Period (dph)	Rotifer (2-18)		*Artemia* (19-30)		Live feed (2-30)		Dry feed (31-45)	
System	RAS	FTS	RAS	FTS	RAS	FTS	RAS	FTS
Daily specific growth rate (%)	7.7 ± 1.3	6.4 ± 1.2	15.1 ± 1.6	16.5 ± 1.8	9.9 ± 0.6	9.9 ± 0.8	12.2 ± 5.2	8.6 ± 2.7
Larval survival (%)					11.5 ± 1.4	2.3 ± N.D.	65.6 ± 6.4	50.0
Replicate tanks					(n = 9)	(n = 4)	(n = 3)	(n = 1)

4 Discussion

4.1 Development of the microbial community

According to the criteria used to define a more stable and K-selected microbial community, the RAS generally offered a more beneficial microbial environment for the larvae than the FTS. The RAS apparently maintained a more stable composition of the microbial community over time and showed less variability between replicate fish tanks. The species richness of bacteria increased in both systems after the first week post hatching and thereafter remained higher and less variable in the RAS, which suggests the development of a more even and diverse structure of the microbial community. The FTS showed proliferation of opportunists and low species richness compared to the RAS during the *Artemia* phase. The water treatment regime explained most of the variation in microbial composition during the live feed period and a ten times lower variation in the composition of bacteria was observed in the RAS compared to the FTS. This stresses the impact of water treatment for hatchery management.

Two different processes can contribute to elevated stability of the microbial composition in RAS compared to FTS. First, RAS may passively conserve the microbial composition simply by retaining water in the system for a longer period of time, leaving less chance of random invasion. Secondly, RAS operation may secure a more even supply of organic matter, i.e. exert a more constant selection pressure on microbes, which results in a more stable microbial composition. Significantly different and presumably more beneficial microbial communities in RAS compared to FTS have been described in other publications both in water (Verner-Jeffreys *et al.*, 2004) and in larvae (Fjellheim *et al.*, 2007, Fjellheim *et al.*, submitted).

Generally higher abundance of bacteria was found in water of RAS than FTS, with both CFU and total bacteria cell counts about one order of magnitude higher in the RAS. The composition of the microbial community seems to be more important for larval performance than absolute bacteria numbers as long as numbers are not extreme (Munro *et al.*, 1995, Verner-Jeffereys *et al.*, 2004). Peaks in the number of bacteria were observed in both systems the first week, but this was mainly a quantitative reaction with little alteration of the microbial composition. Initial proliferation of bacteria as a response to increased CC due to hatching water, algae and rotifers is a well known phenomenon in larval rearing (Skjermo *et al.*, 1997, Salvesen *et al.*, 1999). The composition of the microbial community differed more between systems later in the live feed period when higher water exchange rates in the tanks resulted in

a stronger impact of the external water treatment, and there had been ample time for the microbial community to mature.

A low fraction of cultivable bacteria (CB) may suggest a high level of specialised bacteria with a narrow niche width characteristic of a more mature community. Because of this CB has been used to describe the state of maturation of the microbial community (Skjermo *et al*., 1997). An increase in CB may also be seen as a change from a less active and oligotrophic way of life to a more an active, rapidly growing state (Ruby and Morin, 1979). CB may therefore be a less useful criterion to compare the relative maturation of systems with very different loads of substrate. In the RAS the fraction of cultivable bacteria increased during the *Artemia* period, resulting in a higher and more varying CB than in the FTS. In this case, higher abundance of bacteria and higher CB may be the result of a higher microbial CC and faster DOC turnover rate in the RAS and is not necessarily a sign of a less matured community. Higher CB was also observed in microbially matured water compared to filtered water during the *Artemia* period in an experiment with turbot larvae, *Scophthalmus maximus* (Salvesen *et al.*, 1999). CB has been found to increase with increasing C/N ratio and from inlet to outlet in a marine biofilter (Michaud *et al*., 2006, 2009).

Significantly less variability in the composition of the microbial community was found between parallel fish tanks for RAS than FTS. Whether the higher microbial reproducibility was caused by an overall more matured state, strong influence of the biofilter on the development of the microbial environment of the tanks or due to the possibility of sharing bacteria through the RAS water treatment circuit is hard to conclude when no strong disinfection unit was included in the RAS. This may, however, reflect a more random and unstable environment in the FTS, giving different microbes a foothold in parallel tanks.

4.2 Physiochemical water quality

Although both systems had an acceptable physiochemical water quality during the live feed period, RAS rearing water always contained more nitrogenous waste, particles and dissolved organic matter. In addition pH decreased in the RAS during the *Artemia* period, reflecting higher CO_2 levels and nitrification. Moderate ozonation to an ORP of about 300-350 mV as was used in this experiment is common and considered safe for marine fish in RAS, although some production of toxic bromate has been demonstrated at this level (Tango and Gagnon, 2003). Ozonation to 350 mV do not represent efficient disinfection in a marine RAS (Hsieh *et*

al., 2002), but has been shown to generally improve water quality (Kobayashi *et al.*, 1993, Tango and Gagnon, 2003) leading to increased fish survival and growth (Ozawa *et al.*, 1991, Reid and Arnold, 1994). The ORP was slightly higher in the RAS rearing tanks the first week, which is empirically considered positive. Little is known about how an ORP, i.e. rest oxidants from ozonation, at this level is affecting the fish larvae or the composition of the microbial community.

The pH was more affected in the RAS from the *Artemia* period and onwards, but both systems showed significant water acidification towards the end of the experiment despite relatively low fish density. The pH-level was kept within tolerable levels for the larvae (Brownell, 1980, Poxton and Allouse, 1982, Parra and Yufera, 2002) and nitrifying bacteria (Watson, 1971), although it may have been suboptimal for both larvae and nitrifiers in the dry feed period.

A quantitative predominance of small particles was observed in the systems, consistent with what is commonly observed for hatchery waters (Rueter and Johnson, 1995). As expected, there was also an accumulation of suspended solids in the RAS, consistent with reports from other such systems (Chen *et al.,* 1993, McMillan *et al.*, 2003, Patterson and Watts, 2003a,b). The effects of turbidity in fish culture water depend on the nature and concentration of the suspended particles. Addition of several species of microalgae, concentrated microalgae paste or ceramic clay to fish tanks during early start feeding has been shown to have beneficial effects on the performance of marine fish larvae (Howell, 1979, Naas *et al.*, 1992, Reitan *et al.*, 1993, 1997, Salvesen *et al.*, 1999, Attramadal *et al.*, submitted b). For some anadromous and fresh water fish high concentrations of suspended particles have been shown to cause stress and reduce disease resistance (Redding *et al.*, 1987, Bullock *et al.*, 1994, Lake and Hinch, 1999). Organic particles are sources of bacteria substrate and contribute to oxygen consumption and waste production when degraded. The particle size distribution of the algae paste added to tanks during the rotifer phase suggested that a great part of particles in the water of both systems were algae cells. Even with a well working protein skimmer, RAS tanks had on average more than twice the concentration of particles than the FTS during the rotifer period. It is difficult to conclude if this may have been an advantage for the RAS larvae compared to those in the FTS (Naas *et al.*, 1992). In the *Artemia* and dry feed periods, it may be assumed that particles are of feed origin either directly or through defecation. In this case, particle concentration more directly represents the load of organic matter.

Atlantic cod larvae have a high reliance on amino acids as fuel for energy dissipation, which leads to high excretion of ammonia during the larval phase (Finn *et al.*, 2002). In addition, microbial degradation of uneaten feed contributes to the nitrogenous load (Ellner *et al.*, 1996). In FTS ammonia is diluted, whereas RAS depend on bacteria mediated conversion to nitrite and further nitrate in the biofilter. Both un-ionised ammonia and nitrite are toxic to marine fish larvae and juveniles at low concentrations, with sublethal effects like reduced growth rate (Brownell, 1980, Handy and Poxton, 1993, Parra and Yufera, 1999, Foss *et al.*, 2004, Siikavuopio and Sæther, 2006, Björnsson and Olafsdóttir, 2006). As expected, nitrogenous waste products were low or undetectable in the FTS. Levels of un-ionised ammonia and nitrite measured in the RAS were lower, but in a comparable range of that found in other studies of marine nursery RAS (Olivar *et al.*, 2000), and considered safe (Poxton and Allouse, 1982, Foss *et al.*, 2004, Siikavuopio and Sæther, 2006). The initial peak of ammonia in tanks of both systems the first week after hatching may be explained by the low exchange rate of tank water during this period, allowing accumulation. The ammonia to nitrite conversion step seemed to work satisfyingly in the RAS. However, an accumulation of nitrite indicated a slower establishment and response of the nitrite to nitrate conversion step, as typically seen for marine biofilters (Manthe and Malone, 1987). The nitrite oxidation reaction step was not absent, however, as accumulation of nitrate was observed. A stable nitrifying biofilm is harder to maintain in systems with cold (Wortman and Wheaton, 1991, Zhu and Chen, 2002) and saline water (Nijhof and Bovendeur, 1990, Chen *et al.*, 2003). In addition, larval rearing systems are operating with low, but exponentially increasing levels of substrate as the fish grow, which further complicates the operation (Gutierrez-Wing and Malone, 2006). Zhu and Chen (1999) found that at 27.2°C, the mean minimum TAN concentration needed to support a steady-state nitrification biofilm was 0.07 mg L^{-1}, which is in the range of the TAN concentration observed in the RAS in this experiment, but a higher temperature.

4.3 Performance of fish

Complex factors are expected to influence the performance of fish, including levels of waste products, variability of the physiochemical environment and microbial composition. RAS operation leads to waste accumulation, but may apparently stabilise the microbial environment. Microbial maturation has been shown to improve marine larval performance in the early stages of production (Vadstein *et al.*, 1993, Skjermo *et al.,* 1997, Salvesen *et al.*, 1999). In total, it seemed as fish larvae reared in the RAS performed at least as well as those reared in the FTS. RAS larvae initiated exponential growth earlier, showed an apparent higher

survival and performed better during the stressful transition to dry feed. Early initiation of exponential growth is considered to reflect good quality of marine larvae (Reitan *et al*., 1993, Skjermo *et al*., 1997). The physiochemical water quality was generally considered inferior and more varying in the RAS. Few bacteria DNA samples during the first 10 dph make resolution low for the microbial composition for this period. It is consequently hard to conclude whether differences in microbial composition, or other factors like slightly higher ORP or concentration of algae, may be the principal cause of the early superior growth in the RAS.

The FTS larvae caught up with the dry weight of RAS larvae during the *Artemia* period. Mortality was high in the FTS, and higher growth rates may have been a result of compensatory growth due to density dependent factors and/or size selective mortality caused by smaller individuals being inferior to cope. The frequently observed negative correlation between larval growth and density is a well known phenomenon resulting from a situation of more available feed, improved water quality, less stress and generally better conditions if the survivors are few (Paller and Lewis, 1987, Baskerville-Bridges and Kling, 2000). Cannibalism may also contribute to this pattern, but was not observed in this experiment. Specific growth rates obtained were comparable to reported values from comparable experiments (Puvanendran and Brown, 2002).

Larval growth slowed down and mortality increased in both systems as the fish faced the challenge of accepting dry feed. The physiochemical water quality deteriorated in both systems during the dry feed period, but this was more pronounced in the RAS. The metamorphosis and the transition from live prey to formulated feed are often challenging periods in hatcheries (Sahin 2001, Ustundag *et al*., 2002). RAS larvae coped better during this stressful transition, which may reflect better conditions the first days after hatching.

4.4 Implications

Compared to the FTS, the RAS rearing environment possessed a lot of the qualities characteristic for microbially matured water, including a more stable microbial composition over time, a higher species diversity of bacteria and periodically a lower fraction of opportunists (Vadstein *et al*., 1993, Salvesen, 1999). RAS as a microbial control strategy is particularly applicable during the live feed period when the sensitivity of larvae is high, the biomass of the fish is low and the production of waste is limited. Even if the water exchange

rates are low, waste do not have to accumulate to problematic levels for an extended period of time, maybe the entire live feed period, as shown in this experiment and reported by Olivar *et al.* (2000). The potential of improving physiochemical water quality in the RAS suggests that there is room for further increase in productivity and quality of larvae.

Compared to metamorphosed juveniles, newly hatched marine larvae are sensitive to infections and fluctuations in physiochemical water quality. The most important feature of the biofilter the first days of larval rearing may not be as a nitrifying unit, but rather as a K-selection unit for heterotrophic bacteria which provides microbial stabilisation of the rearing water. When the aim is to stabilise rearing conditions rather than saving water and energy, the degree of water reuse may be balanced to optimise physiochemical water quality and microbial control. How high the degree of recirculation has to be to maintain a microbial stabilising effect is not known. At some point down the production line, however, fish are more robust and addressing other challenges than microbial control become increasingly more important, i.e. oxygen demand and waste removal.

Little is known about how disinfection doses and different disinfection methods like ozonation and UV-irradiation affect the development of the microbial community and the degree of maturity in RAS. Other strategies for microbial control may have the potential to work in synergy with RAS maturation, like addition of probiotic bacteria to the rearing water.

5 Conclusions

The marine nursery RAS with moderate ozonation developed and maintained a significantly different and more even and stable microbial community composition with a higher diversity of species and periodically a lower fraction of opportunists compared to a traditional FTS with the same feed and rearing regime. Atlantic cod larvae reared in the RAS performed equally well or better than their FTS siblings despite being exposed to an apparent inferior physiochemical water quality. This supports the suggestion that RAS operation may have a beneficial stabilising and maturing effect on the development of the microbial community compared to FTS. RAS are widely used to reduce water consumption and energy use, and to control waste emission. This initial investigation suggests that RAS may also be used as a strategy in the microbial management of marine hatcheries. However, further experiments are required to verify this, and to optimise design and operation for obtaining improved microbial water quality.

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Paper 2

The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae

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Abstract

Marine fish larvae are sensitive to infections by opportunistic bacteria. Procedures like disinfection and pulse feeding may destabilise the microbial community and promote r-selection and proliferation of opportunists in intensive rearing tanks. Recirculation aquaculture systems (RAS) have been hypothesised to stabilise and mature the microbial community, creating a more beneficial environment for the larvae during the live feed period. Two marine RAS with Atlantic cod larvae (*Gadus morhua* L.) with either moderate ozonation (RAS O_3) or high intensity UV-irradiation (RAS UV) were compared with a flow-through system (FTS). The two RAS developed a different and more stable composition of the microbial community than the FTS. The RAS O_3 had a more mature and stable microbial community than the RAS UV. The density and the activity of bacteria were higher in the rearing tanks than in the in-flowing water in the RAS UV system, whereas for the RAS O_3 system densities and activity of bacteria were similar, indicating low disinfection efficiency with moderate ozonation. Atlantic cod larvae reared in the RAS O_3 showed the best survival and growth, whereas the RAS UV larvae performed equally well or better than their siblings in the FTS. This was in spite of the fact that the physiochemical water quality of the two RAS was inferior to that of the FTS. The agar-based method used to quantify opportunists may be too general to capture important differences in the microbial community of the rearing water. For the future, molecular methods could be used to identify which functional groups or species of bacteria are contributing to the observed RAS effect. Our results support the hypothesis of RAS as a microbial control strategy during first feeding of larvae. However, a RAS for marine larvae should probably not include strong disinfection because it leads to a reduction in bacterial numbers, which is likely to result in a destabilization of the microbial community.

1 Introduction

Pelagic marine fish larvae hatch at an early developmental stage. The first weeks after hatching they depend on the general immune system, and larvae are sensitive to infections. Most infections in young marine larvae are assumed to be caused by opportunistic bacteria becoming pathogenic when the resistance of the host is lowered by environmental stress. A high fraction of opportunistic bacteria in the rearing water has been shown to reduce the performance of marine fish larvae (Vadstein *et al.*, 1993, Skjermo *et al.*, 1997, Salvesen *et al.*, 1999, Skjermo and Vadstein, 1999). Efficient strategies to increase microbial control may help to increase and stabilise the production of marine juveniles. The use of recirculating aquaculture systems (RAS) has been suggested to stabilise and improve the microbial environment for marine larvae during first feeding, but little is known about how disinfection doses and different disinfection methods like ozonation and UV-irradiation affect the development of the microbial community in RAS (Attramadal *et al*., submitted).

The abundance of bacteria and the composition of the microbial community of the rearing water depend on the supply of bacteria and organic matter, together with selective forces in the tank and in the sources (Vadstein *et al.,* 1993, 2004). The main contributors of bacteria to rearing tanks in marine hatcheries are live feed (Skjermo and Vadstein, 1993, Olsen *et al*., 1999, Blancheton and Canaguier, 1995), algae (Salvesen *et al*., 2000) and intake water, as eggs are commonly surface disinfected (Salvesen and Vadstein, 1995). According to the ecological r/K-theory (MacArthur and Wilson, 1967), r-selected opportunistic species have the ability to grow fast and are typically favoured in perturbed or unpredictable environments with little competition (e.g. a high substrate supply per capita). K-strategists, on the other hand, are specialists that compete better for limited resources, and are favoured in stable environments with communities close to the carrying capacity (CC). The CC is the maximum number of bacteria that can be sustained in a system for an extended period of time. CC is defined by density dependent restrictions like availability of resources, which typically is supply of dissolved organic matter (DOM) for the heterotrophic bacteria.

Procedures like disinfection and pulse feeding may destabilise the microbial community in intensive marine hatcheries (Salvesen, 1999, Salvesen *et al*., 2000). Following a perturbation, opportunistic pioneer species of bacteria are the first to colonise the free niches that are created due to increased supply of resources or decimation in the number of competitors

(Hess-Erga *et al*., 2010). As the population increases to CC and the resources become limited, the r-strategists are gradually out-competed by K-selected species. This succession in the microbial community takes about one week in sea water of 5-18°C (Salvesen, 1999).

The host/microbe interactions in the rearing water may be improved by promoting K-selection to reduce the fraction of opportunists (Vadstein *et al*., 1993). K-selection may be created by maintaining low substrate availability per bacteria (Vadstein *et al*., 1993, Skjermo *et al.,* 1997, Salvesen *et al*., 1999). This strategy involves controlling two important factors: 1. the supply of substrate and 2. the number of bacteria competing for the substrate. Ideally, the supply of dissolved organic matter should be stable and the microbial population should be close to CC to obtain a low fraction of opportunists. RAS may provide a sufficiently long retention time of water to allow the microbial succession to take place at a relatively stable level of organic matter which secures K-selection, and are therefore hypothesised to mature and stabilise the microbial community of the rearing water compared to flow through systems (FTS) (Attramadal *et al*., submitted).

UV-irradiation and ozonation are two common methods used for disinfection of intake water. The water treatment in a RAS may include UV-irradiation to reduce the abundance of bacteria. Ozonation in RAS, however, is in most cases motivated by improvement of the physiochemical water quality rather than disinfection (Summerfelt, 2003, Tango and Gagnon, 2003). Ozone is unsuitable as a disinfection method in marine RAS for several reasons. One is that RAS process water requires high amounts of ozone to inactivate bacteria, as the oxidative power of ozone and residual oxidants is consumed in reactions with organic matter and other components of the rearing water. Another reason is that several compounds formed when high doses of ozone are added to sea water are highly toxic to fish and live feed (Ozawa *et al.*, 1991, Davis and Arnold, 1997, Grotmol and Totland, 2000), although some of the residual oxidants can be reduced by passing the water through activated carbon filters (Ozawa *et al*., 1991, Kobayashi *et al.*, 1993).

The level of ozonation of culture water is commonly controlled by continuous measurements of the oxidation reduction potential (ORP), which is an indirect measure of the concentration of free radicals in a solution. Moderate ozonation to an ORP of about 300-350 mV is common and considered safe for marine fish in RAS, although some production of toxic bromate has been demonstrated at this level (Tango and Gagnon, 2003). Ozonation to 350 mV does not

represent efficient disinfection in a marine RAS (Hsieh *et al*., 2002), but has been shown to improve the physiochemical water quality (Kobayashi *et al*., 1993, Tango and Gagnon, 2003) leading to increased fish survival and growth (Ozawa *et al*., 1991, Reid and Arnold, 1994).

The disinfection effects of UV-irradiation and ozonation are strongly influenced by turbidity and the presence of inorganic, as well as organic particles in the water that may protect microorganisms from inactivation (Hess-Erga *et al*., 2008). UV-transmittance is reduced by turbidity and may be blocked by particles, whereas the oxidative power of ozone and rest oxidants can be reduced due to degradation of disinfectant at the surface of suspended solids and the rate limited transport into particles (Perrins *et al*., 2006). During the rotifer period, addition of microalgae to the culture water has a beneficial influence on survival and growth of marine fish larvae (Howell, 1979, Naas *et al*., 1992, Reitan *et al*., 1993, Salvesen *et al*., 1999, Lazo *et al*., 2000). During the "green water" period, with high turbidity due to small suspended particles, the disinfection efficiency in a RAS is probably low for both UV-irradiation and ozonation.

UV-irradiation is a physical disinfection method, whereas ozone is a chemical agent. Most likely, the dose and mechanism of the hygienic barrier may influence microbial selection, stabilization, maturation and the development of the microbial community in a RAS over time. Because RAS are commonly operated at low or moderate levels of ozonation, but with high intensity UV, the two methods are likely to represent different levels of disinfection efficiency.

Here we present a study of the effects of UV-irradiation *versus* moderate ozonation on the level of maturation, K-selection and stability of the microbial community in the rearing water of two marine RAS with Atlantic cod (*Gadus morhua* L.), and we compare them with a FTS. The two RAS were identical apart from including either ozonation to 350 mV and two active carbon filters or UV-irradiation. Both the UV-irradiation and the ozonation happened before the water was biofiltered. The growth and survival of fish reared in the three systems were compared.

2 Materials and methods

2.1 Experimental setup

Figure 1 shows a flow scheme of the systems. Intake water (70 m depth) from Trondheimsfjorden was pumped through a sand filter (Triton TR-140, Pentair Inc., USA) and a protein skimmer (Helgoland 500, Erwin Sander Elektroapparatebau GmbH, Germany) before it was led to a reservoir with a vacuum aerator and a heating/cooling system (Carrier Corp., USA).
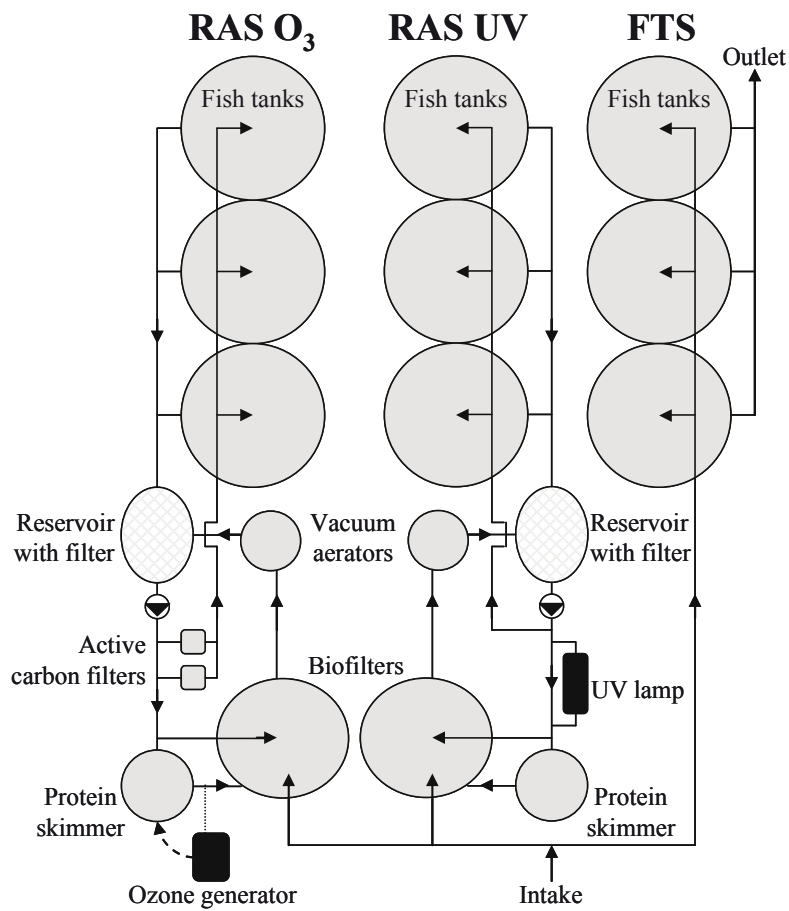

Fig. 1: Flow scheme of the two RAS and the FTS (not to scale).

Both RAS included a 150 L pump sump with a removable cross flow filter of 50 μm during the rotifer period and 150 μm thereafter (Termosveis AS, Norway) and a titanium pump (CRT8-1, Grundfos, Denmark). The circulation pumps were controlled by water level sensors in the pump sumps. Both RAS included a protein skimmer (hydraulic retention time (HRT) 0.10 h), Outside Skimmer III P, Erwin Sander Elektroapparatebau GmbH, Germany) and a cylindrical moving bed biofilter (HRT 0.15 h). The biofilters (each 700 L) were 30 % filled with KMT Kaldnes K1 Media (Kaldnes Miljøteknologi AS, Norway) and had strong aeration from pressurized air through a perforated air tube in the bottom circumference. Both biofilters were conditioned by addition of 18 g formulated fish feed (Gemma Micro Diamond 300, Skretting, Norway) and 12 mg NH_4-N L^{-1} d^{-1} for 2 months prior to the experiment. Water from the biofilters was returned to the pump sumps through a 2 m high 32 cm diameter vacuum aerator containing parts of BIO-BLOK medium (EXPO-NET, Denmark). The RAS O_3 system included ozonation from a generator (A1000, Erwin Sander Elektroapparatebau GmbH, Germany) in the protein skimmer. The oxidation reduction potential (ORP) was measured continuously by a platinum electrode in the protein skimmer outlet connected to a Redox-Potential Meter (Sander Aquarientechnik, Uetze-Eltze) and secured 350 mV. The water from the protein skimmer flowed into the biofilter. The water in the RAS O_3 passed two parallel activated carbon filters (1 L each) before it reached the fish tanks. The RAS UV system included a UV-reactor (A10-PE, Wedeco, Germany) on a side stream of water (1.5 m^3 h^{-1}) going to the protein skimmer and biofilter.

Intake water (FTS) or water from the pump sumps (RAS) was pumped to three 1.8 m^3 black, flat bottom fish tanks in each system (Strandvik Plast AS, Norway). Efficient circulation was secured in the tanks by 90 cm intake pipes with 2 mm holes each 7 cm. Tank outlets were central cylinders covered with nylon net (400 μm in the rotifer period, thereafter 800 μm) connected with a pipe to the side drain during the live feed period. Air diffusors attached to the cylinders secured turbulence at the outlet net. During the dry feed period the tanks were operated with bottom drains with a grid. Tanks were equipped with automatic cleaning arms on the bottoms and surface skimmers to collect wastes gathering in the surface film.

2.2 Rearing regime

The rearing regime is presented in Table 1. Water was added algae and allowed to stabilize in the fish tanks of each system for three days prior to egg transfer. Atlantic cod (*Gadus morhua*) eggs (63°d) were received from Havlandet Marin Yngel AS. The eggs were

disinfected in 400 ppm glutaric dialdehyde for 6 minutes upon arrival (Salvesen and Vadstein, 1995), and incubated at 7.5°C in hatching incubators to about 85°d, when they were transferred to the experimental tanks at a final density of 60 individuals L^{-1}. 99 % hatching success was achieved. Larvae were hatched and maintained in darkness the first 3 days, then with continuous light of 6-65 lux on the water surface in the tank periphery and centre, respectively (Quantum Radiometer LI-185B, LI-COR inc., USA). During the live feed period, gentle central aeration contributed to circulation. Water exchange rates were gradually increased from 1 to 6 tank volumes d^{-1}, limited by the maximum capacity of the pumps of the two RAS. Intake water (200 L d^{-1}) was added to the biofilters of the two RAS during the *Artemia* and the dry feed period. The total water volume of each RAS was 5500 L. Semi-continuously cultured *Isochrysis galbana* was transferred to a reservoir each morning and was continuously pumped to the fish tanks (100 L d^{-1} to each tank, electromagnetic metering pumps, EW, IWAKI, Japan). The goal was to achieve an algae concentration of about 1.5 mg C L^{-1} in the rearing tanks, but under-dimensioned production resulted in an algae supply only half of this every other day. Batch cultured rotifers (*Brachionus* ' Nevada') fed *Nannochloropsis oculata* algae paste (Reed Mariculture, California), Baker's yeast and Marol-E (rotifer and *Artemia* oil emulsion enrichment, SINTEF, Trondheim) were distributed to the fish tanks from day 2 to day 23 ph. *Artemia* nauplii were hatched from INVE EG cysts with Hatch Control (INVE, USA) and short time enriched with Marol E before they were fed to the fish (day 20 to 31 ph). Live feed organisms were washed in sea water and concentrated before they were fed to the larval tanks four times a day to obtain a total tank concentration of 5 individuals mL^{-1}. Feeding dispensers (Raunes micro feeder, Sterner Fish Tech, Norway) provided 4-8 g dry feed (Gemma Micro Diamond 300, Skretting, Norway) to each tank every 15 min from day 31 to 46 ph.

Table 1: Rearing regime.

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2.3 Analytical procedures

Water quality and bacterial load and composition was measured or sampled at random points in each fish tank before tanks were cleaned and the fish were fed. Water for particle counts, fluorescence microscopy, DNA extraction and agar plating was screened (50 μm) to exclude live feed organisms before processing.

2.3.1 Growth and survival of fish larvae

Larvae sampled for carbon biomass analysis (n = 12 from each tank in each system) were sacrificed with an overdose of Tricaine Methanesulfonate (MS222), rinsed in fresh water and transferred to individual tin cups (Mikro Kemi AB, Sweden) and dried (60ºC, 48 h). Larvae sampled during the live feed period were thereafter analyzed in a CHN Elemental Analyser 1106 (Carlo Erba Instruments, Italy). Individual dry weight (DW) of larvae was calculated from measured carbon content using a conversion factor of 2.34 (Reitan *et al.*, 1993). Larvae sampled during the dry feed period were weighed after drying. Daily percentage specific growth rate (% SGR) was calculated from larval dry weight (DW) for the time period t_1 to t_2 according to Equations 1 and 2:

$$SGR\ (d^{-1}) = (\ln DWt_1 - \ln DWt_1) / (t_1 - t_2) \tag{1}$$

$$SGR\ (\%\ d^{-1}) = (e^{SGR-1}) \times 100\ \% \tag{2}$$

The system difference in temperature (± 0.15 °C) was corrected for by comparing larval size at the same day degree. This was calculated from individual dry weight and SGR for each sample time according to equation 3 and 4:

$$\text{Ln } DW_{corr\ low\ temp} = \ln DW_{ind} + SGR_{low\ temp} \times d°_{average} / °C_{average\ low\ temp} \tag{3}$$

$$\text{Ln } DW_{corr\ high\ temp} = \ln DW_{ind} - SGR_{high\ temp} \times d°_{average} / °C_{average\ high\ temp} \tag{4}$$

In order to compare the density and hence survival of larvae in each tank during the live feed period, a digital camera was used to take pictures of a volume in the upper water column (Shaw, 2006). To get a clearly delimited volume, both the camera and a white plastic board were attached to a metal pole at a fixed distance from each other and held so the camera pointed down toward the surface and the white board lay horizontally below the surface 20-30

cm from the tank wall. The larvae may be more concentrated in some parts of the tank compared to others. However, on the assumption that the distribution of larvae is similar between tanks, the measurement can still give information on the development with time and the relative differences between groups.

Total numbers of surviving fish were determined at 46 dph after termination of the experiment with an overdose of MS222. Mortality was recorded daily from day 31 ph by sub-sampling dead larvae removed during cleaning. Estimated survival at the end of the live feed period (30 dph) was calculated from the recorded daily mortality during the dry feed period and survival at termination of the experiment. The recorded mortality represented a minimum each day due to the rapid degradation of dead larvae. Therefore, the survival through the live feed period was related to the survival registered in three FTS control tanks from a parallel experiment with the same sibling group and rearing regime that was terminated 20 dph, and to the relative decline of larvae registered by photography during the live feed period.

2.3.2 Analysis of microbial communities

The number of colony forming units (CFU) was determined from growth on M-65 seawater agar (Salvesen and Vadstein, 2000). Three 10-fold dilutions were plated from each sample, and each dilution was plated in duplicate. Samples were incubated in darkness at $12 \pm 1°C$. Total CFU were calculated as the average of colonies after 14 days of incubation. Plates containing 30-300 colonies were preferably counted. Because r-strategic opportunists are characterised by high maximum growth rates in contrast to K-strategic specialists, the fraction of fast-growing bacteria of total CFU was used as a measure of the relative presence of opportunistic bacteria (Skjermo *et al.*, 1997, Salvesen and Vadstein, 2000). In this paper, the term opportunistic bacteria is used about results to denote the CFU emerging the first two days of incubation on M65 agar as described by Salvesen and Vadstein (2000).

The total numbers of bacteria were determined by fluorescence microscopy (Hobbie *et al.*, 1977). Samples were fixed with formaldehyde (0.7 % final concentration) and stored dark at 4ºC. Two ml sample was diluted with 3 ml milli-Q water and vacuum filtered onto black polycarbonate filters (0.22 μm 25 mm diameter, Poretics Corp., California) on supporting mixed cellulose ester membrane filters (0.45 μm 25 mm diameter, Whatman, UK). Three ml of DAPI (4.6-diamidino-2-phenylindole, 1 mg L^{-1} dH_2O) was added to stain the bacteria for 10 minutes (Porter and Feig, 1980). The dye was removed by filtration and the filters stored

dark and dry. Stained bacteria were counted in a fluorescence microscope (Zeiss Axioplan 2, Germany) at 1250× magnification using UV excitation. A minimum of 250 individual bacteria in at least 5 different random squares on the filter were counted for each sample. The fraction of cultivable bacteria (CB) was calculated as the percentage total CFU of total cell counts. The CB may be used as an indicator of the relative amount of K-strategic specialists present (Skjermo *et al.*, 1997).

The production of heterotrophic bacterial biomass was estimated from the incorporation of L-[4,5-^{3}H]Leucine (GE Healthcare, UK) into macromolecules (protein biosynthesis) as described by Kirchman (1993). A lukewarm leucine solution (1 part ^{3}H leucine and 9 parts leucine) was added at a final concentration of 40 nM to 1.2 mL water samples in Eppendorf tubes. The samples were incubated for 1 h at room temperature, terminated by addition of trichloroacetic acid (TCA, 5 % final concentration), processed by the centrifugation method (Smith and Azam, 1992), and quantified in a liquid scintillation counter (Wallac 1410, Perkin Elmer Life Sciences, USA). Incubations were done with water from each tank and from the inflowing water for each system, using three replicate samples and one control (inactivated with TCA before incubation). Calculations of bacterial production were done according to Kirchman (1993).

Water samples (50 ml) for characterization of bacterial communities using PCR/T-RFLP were filtered through sterile 0.2 μm 2.5 cm^2 hollow fibre syringe filter for aqueous solutions (DynaGard, Microgon Inc., California) to retain bacteria. Excess water was removed by gentle centrifugation, and filters were stored at -20ºC. DNA was extracted using a DNeasy tissue kit from Qiagen following the Gram negative bacteria protocol. DNA extracts were stored at -20ºC. The targeted region of 16S rRNA was amplified in a 7500 Real Time PCR System (Applied Biosystems, California) using the fluorescence labelled forward primer 8f (5'-FAM-AGA GTT TGA TC(AC) TGG CTC AG-3') and the reverse primer 517r (5'-ATT ACC GCG GCT GCT GG-3'). The 25 μl reaction mixture included Power SYBR Green 2×PCR master mix (12.5 μl, Applied Biosystems, California), primers (2 μM, 2.5 μl each), bovine serum albumin (10 mg L^{-1}, 0.75 μl, Sigma, USA), dH2O (1.75 μl) and 5 μl DNA template. PCR amplifications were run in duplicates for each sample with an initial denaturation step at 95°C for 10 min followed by a variable number of cycles of denaturation at 95°C (15 s), annealing and extension at 60°C (1 min). Samples were run the required

number of cycles to end the PCR in the late logarithmic DNA amplification phase (20 to 32 cycles). A negative control was included in each PCR run.

Duplicate PCR products were mixed and cut with the restriction enzyme AluI (New England BioLabs, USA) at 37°C for one hour. The cutting mixture contained PCR product (10 μl), 10×NEBuffer 4 (3 μl, New England BioLabs, USA), AluI (1 μl) and sterile dH$_2$O (16 μl). Digested PCR products were precipitated with ethanol (96 %) and 3M NaAc and the resulting DNA pellet was washed with 70 % ethanol before it was air dried at 37°C and stored at -20°C. Samples were prepared for analysis by addition of 12 μl formamide and 0.15 μl GenescanTM 500 LIZTM Size Standard (Applied Biosystems, California) to the digested PCR product. Samples were denatured at 95°C for 6 min and then rapidly chilled on ice. The lengths of TRFs were determined with an ABI Prism 3130xl Genetic analyser (Applied Biosystems, California), and the peak area data were standardized by the variable percentage threshold method (Osborne *et al*., 2006). The divisor that resulted in the weakest relationship between number of peaks remaining and initial total area was used for further calculations.

2.3.3 Physiochemical water quality

The pH and oxygen concentration was measured daily with portable electrodes (Oxyguard, Denmark). Nitrite nitrogen (NO$_2$-N) and nitrate nitrogen (NO$_3$-N) concentrations were analysed in a Skalar SA2000/4000 Segmented Flow Analyser (Skalar Analytical B.V., The Netherlands). Total ammonia nitrogen (TAN) was analysed by the Norwegian Standard method (NS 4746, 1975). Unionized ammonia (NH$_3$-N) concentration was calculated from TAN and pH considering temperature and salinity. The oxidation reduction potential (ORP) was measured by platinum redox electrodes (Erwin Sander Elektroapparatebau GmbH, Germany). Dissolved organic carbon (DOC) samples (45 mL) were immediately vacuum filtered through ignited (480ºC, 2 h) 0.7μm, 25mm diameter GF/F glass microfiber filters (Whatman International Ltd., England). The filtrate and filters were stored at -20ºC. The filtrate was analysed in a Tekmar-Dohrmann Apollo 9000 TOC-analysator (Teledyne Tekmar, USA). Inorganic CO$_2$ was removed from filters with hydrochloric acid vapour (37 %, 20 min). Each filter were transferred to a tin cup (Säntis Analytical AG, Switzerland) and analyzed in a HN-S/N Elemental Analyser 1106 (Carlo Erba Instruments, Italy).

2.4 Statistical analysis

Mean ± standard error of the mean (SE) or standard deviation (SD) is presented. Statistical analysis was performed at the 95 % confidence level (p < 0.05). Data for larval dry weight were $\log_{10}$ transformed to secure a stable variance, and tested for differences by one-way ANOVA and t-tests in SPSS 16.0 (SPSS Inc., Chicago). SGR standard error was calculated by linear regressions of $\log_{10}$ transformed individual DW data (Sigmaplot, Systat Software Inc., USA). Levene's test for equality of variances and one-way ANOVA with Tukey's-b test post hoc were used to compare physiochemical and microbial water quality variables between the three systems. A non-parametric test (Kruskal-Wallis) was used when the Levene's test for equality of variances was significant.

The mean centered data of the T-RFLP fingerprints from the samples were compared in a principal component analysis (PCA). R (http://cran.r-project.org/) was used to plot the scores plot in Figure 3. The T-RFLP data were also analysed with two other multivariate analysis methods: detrended correspondence analysis (DCA) and multidimensional scaling (MDS), which both gave similar conclusions as the PCA (data not shown). The standard deviation (SD) of the PC1 and PC2 score values was used as a measure of the change in the T-RFLP fingerprint. The $SD_{PC1} \times SD_{PC2}$ value is directly proportional with the amount of change observed. PCA was performed using the PLS toolbox v5.2 from Eigenvector research under Matlab v7.2.

The species richness, i.e. the abundance of T-RFLP operational taxonomic units (OTU), was used to give some idea of the relative distribution and evenness of the dominating species of bacteria (Blackwood *et al*., 2007). Shannon's diversity index (H') was calculated for the T-RFLP profile of each sample.

3 Results

3.1 Physiochemical water quality

Table 2 shows a summary of the physiochemical water quality in the three systems during the periods with different feed. Temperature was on average 11 ºC, and salinity was 34.5 ppt. The oxygen concentration was similar and high (> 85 % saturation, average 96 %) in all the systems during the live feed period, but decreased to low levels at the end of the dry feed

period. The pH was significantly higher in the FTS during the experiment (p <0.001). The pH declined in both RAS from the *Artemia* period (slopes of -0.04, $r^2 > 0.86$, Sigmaplot), whereas a small reduction in pH was observed in the FTS during the same period (slope of -0.01, $r^2 = 0.55$). The ORP was significantly higher in the FTS than in the RAS UV during the live feed period (p = 0.017). The ORP declined in the dry feed period, reaching lower levels in the two RAS than in the FTS (p = 0.010). The concentration of DOC was generally similar in the two RAS and higher than in the FTS for all feed periods (p < 0.001). The concentration of POC was generally low, but significantly higher in the RAS O_3 and significantly lower in the FTS than in the other treatments (p < 0.001). For uncertain reasons, a higher production of foam was observed in the protein skimmer in the RAS UV than in the RAS O_3, and this may explain the significant difference in the concentration of POC between the RAS systems. As expected, the concentration of nitrogenous waste compounds remained significantly lower in the FTS than in the two RAS during the experiment (p < 0.001 for TAN, NO_2-N and NO_3-N). The development of the concentration of ammonia and nitrate was generally similar in the two RAS. There was a peak in the concentration of ammonia in the rotifer period and a second peak in the dry feed period in both RAS, reaching maximum levels of about 0.2 and 0.6 mg TAN L^{-1} at 10 and 36 dph, respectively. Nitrate accumulated in an exponential manner in the two RAS and reached a maximum level of about 27 mg NO_3-N L^{-1} in the tanks at the end of the experiment. On average, the concentration of nitrite was lower in the RAS UV than in the RAS O_3 during the *Artemia* period, but higher during the rotifer period and about twice as high during the dry feed period, reaching a maximum level of 4.2 mg L^{-1} in one of the replicate tanks 41 dph.

Table 2: Physiochemical water quality in the tanks of the two RAS and the FTS during the different stages of feeding (average ± SE) including temperature, oxygen saturation (O_2), pH, the oxidation reduction potential (ORP), total ammonia nitrogen (TAN), unionised ammonia nitrogen (NH3-N) nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N), dissolved organic matter (DOC) and particulate organic matter (POC). The level of nitrogenous waste products (TAN, NH_3-N, NO_2-N and NO_3-N) and organic matter (DOC and POC) was higher in the two RAS than the FTS. The pH, ORP and oxygen saturation decreased during the dry feed period in all systems, but more in the two RAS than in the FTS.

Period	Rotifer (2-19)			Artemia (20-30)			Dry feed (31-45)		
System	RAS UV	RAS O_3	FTS	RAS UV	RAS O_3	FTS	RAS UV	RAS O_3	FTS
Temperature (°C)	10.0 ± 0.2	10.3 ± 0.2	10.0 ± 0.2	11.9 ± 0.0	12.1 ± 0.0	12.1 ± 0.0	11.8 ± 0.0	12.0 ± 0.0	11.7 ± 0.1
O_2 (%)	97.1 ± 0.3	96.9 ± 0.3	97.0 ± 0.3	95.0 ± 0.4	91.8 ± 0.5	92.3 ± 0.6	77.2 ± 2.4	71.9 ± 2.6	82.1 ± 2.4
pH	7.9 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	7.8 ± 0.0	7.7 ± 0.0	8.0 ± 0.0	7.3 ± 0.0	7.2 ± 0.0	7.8 ± 0.0
ORP (mV)	210 ± 1	224 ± 1	224 ± 1	229 ± 1	238 ± 1	237 ± 1	155 ± 2	155 ± 2	169 ± 1
TAN (µg L^{-1})	154 ± 12	147 ± 11	26 ± 2	123 ± 4	94 ± 2	17 ± 1	311 ± 49	415 ± 57	23 ± 6
NH_3-N (µg L^{-1})	2.0 ± 0.2	2.7 ± 0.2	0.5 ± 0.0	1.7 ± 0.1	1.0 ± 0.0	0.4 ± 0.0	1.2 ± 0.2	1.4 ± 0.2	0.9 ± 0.1
NO_2-N (µg L^{-1})	241 ± 39	141 ± 32	2 ± 0	322 ± 11	715 ± 47	3 ± 0	2390 ± 281	1264 ± 173	6 ± 2
NO_3-N (mg L^{-1})	2.2 ± 0.4	2.4 ± 0.3	0.2 ± 0.0	7.2 ± 0.3	7.2 ± 0.2	0.1 ± 0.0	18.0 ± 3.0	13.0 ± 3.0	0.1 ± 0.0
DOC (mg L^{-1})	6.2 ± 0.4	7.5 ± 0.4	2.9 ± 0.1	9.9 ± 0.3	9.2 ± 0.2	2.4 ± 0.2	10.2 ± 0.4	8.0 ± 0.7	2.5 ± 0.3
POC (mg L^{-1})	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.1 ± 0.0	0.5 ± 0.1	0.9 ± 0.1	0.2 ± 0.0

3.2 Microbial environment

The number of CFU was within the same range in the three systems (p = 0.986), whereas total bacteria cell counts were higher in the RAS tanks than in the tanks of the FTS (p < 0.001, Table 3) and higher in the RAS O_3 than in the RAS UV for the *Artemia* and dry feed period (p = 0.036, Figure 2 a). The percent CB was higher in the FTS during the *Artemia* and dry feed period (p = 0.017). The RAS UV showed a higher fraction of opportunists than the other systems during the *Artemia* period (p = 0.008). For this study, the methods that involved agar plating showed high variation, which made it hard to conclude on differences in CFU and the relative abundance of opportunists between the treatments. Molecular methods may be more useful to more accurately identify specific functional groups of bacteria (for example opportunists). The bacterial biomass production was higher in the tanks of the RAS O_3 than in the other two systems during the *Artemia* and the dry feed period (p < 0.001) and higher in the RAS UV than the FTS during the dry feed period (p < 0.001, Figure 2 b).

The abundance of bacteria in the rearing tanks compared to the water reservoirs of the RAS, is an indicator of the disinfection efficiency (Figure 2 a). The abundance of bacteria in the treated water in the RAS O_3 was 109 ± 12 % of that in the rearing tanks, whereas it was 38 ± 7 % in the RAS UV. Similarly, a comparison of the bacterial production in the reservoir and in the tanks is an indicator of the effect of UV-irradiation and ozonation (Figure 2 b). The biomass production of the bacteria in the treated water was 83 ± 1 % of that of the water in the rearing tanks in the RAS O_3 compared to 43 ± 11 % in the and RAS UV.

RAS UV showed a higher diversity of bacteria (Shannon's diversity index) than the FTS over the experiment (p = 0.006, Table 3). Using terminal-restriction fragments (T-RFs) as an indicator of richness of bacteria suggest lower species richness in the FTS than in the two RAS during the *Artemia* period (p = 0.024). In both RAS the richness increased from start to the end of the experiment.

PCA of T-RFLP data revealed that PC1 and PC2 accounted for 53 % of the variation in the data set (36 % and 17 % for PC1 and PC2, respectively). To evaluate the variation of the microbial composition between the periods with different feed types, the arithmetic mean of the PCA score values within each period was calculated for each treatment (Figure 3). Generally, water treatment separated the sample scores on the PC1 axis, whereas feed

type/time separated the scores along the PC2 axis. The largest difference in the microbial community composition was between the FTS and the RAS O_3 (Figure 3). The SD_{PC1} value is an indicator of the amount of variation in the microbial flora that can be related to water treatment in each system. The FTS showed the most unstable composition of bacteria with an average SD_{PC1} of 0.21 for the four periods (Figure 3). The RAS UV showed 2.3 times less variation in the microbial flora (average $SD_{PC1} = 0.09$), whereas the RAS O_3 had 5 times more stable microbial community with an average SD_{PC1} of 0.04. The SD_{PC2}, which is an indicator of the amount of variation in the qualitative microbial flora that can be related to feed type/time, was similar for the three systems (0.06 for FTS and RAS UV, and 0.05 for RAS O_3).

Fast-growing filamentous bacteria quickly overgrew the FTS tanks during the dry feed period, even if the filaments were removed several times daily (data not shown). Many seemingly healthy larvae were observed trapped and dead in the bacteria filaments. Filaments of bacteria were also observed in both the RAS, but to a very small extent compared to that in the FTS.

Table 3: Characteristics of the microbial community in the rearing water of the two RAS and the FTS (average ± SE). The total bacteria cell counts were done via microscopy. The fast-growing opportunists are shown as relative fractions of total colony forming units (CFU). Cultivability (CB) is the proportion of CFU of the total counts of bacteria. The production of heterotrophic bacterial biomass was calculated from the incorporation of ^3H leucine into macromolecules. Shannon's diversity index and species richness of bacteria was calculated from T-RFLP data.

| Period (dph)	Rotifer (2-19)			Artemia (20-30)			Dry feed (31-45)		
System	RAS UV	RAS O_3	FTS	RAS UV	RAS O_3	FTS	RAS UV	RAS O_3	FTS
Total counts of bacteria × 10^6 mL^{-1}	3.0 ± 0.3	2.9 ± 0.4	1.0 ± 0.1	5.7 ± 0.8	9.9 ± 1.9	1.7 ± 0.2	7.9 ± 1.7	11.4 ± 1.9	2.2 ± 0.4
CFU ×10^5 mL^{-1}	4.7 ± 0.7	5.4 ± 1.1	3.1 ± 0.7	1.1 ± 0.3	1.9 ± 0.2	2.0 ± 0.7	18.9 ± 5.0	16.7 ± 2.9	13.6 ± 3.3
Opportunists (%)	4 ± 1	4 ± 1	7 ± 3	41 ± 10	14 ± 7	7 ± 2	62 ± 17	43 ± 12	60 ± 13
CB (%)	20 ± 4	28 ± 8	34 ± 9	2 ± 0	3 ± 1	11 ± 3	22 ± 4	21 ± 6	57 ± 15
Biomass production (μg C incorporated L^{-1} d^{-1})	4 ± 1	4 ± 1	2 ± 1	4 ± 0	16 ± 3	2 ± 0	41 ± 6	58 ± 12	27 ± 2
Shannon's diversity index	2.8 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	2.9 ± 0.2	2.8 ± 0.4	2.7 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	2.9 ± 0.2
Species richness	44 ± 5	49 ± 4	42 ± 4	47 ± 7	55 ± 8	29 ± 3	65 ± 11	66 ± 14	59 ± 10
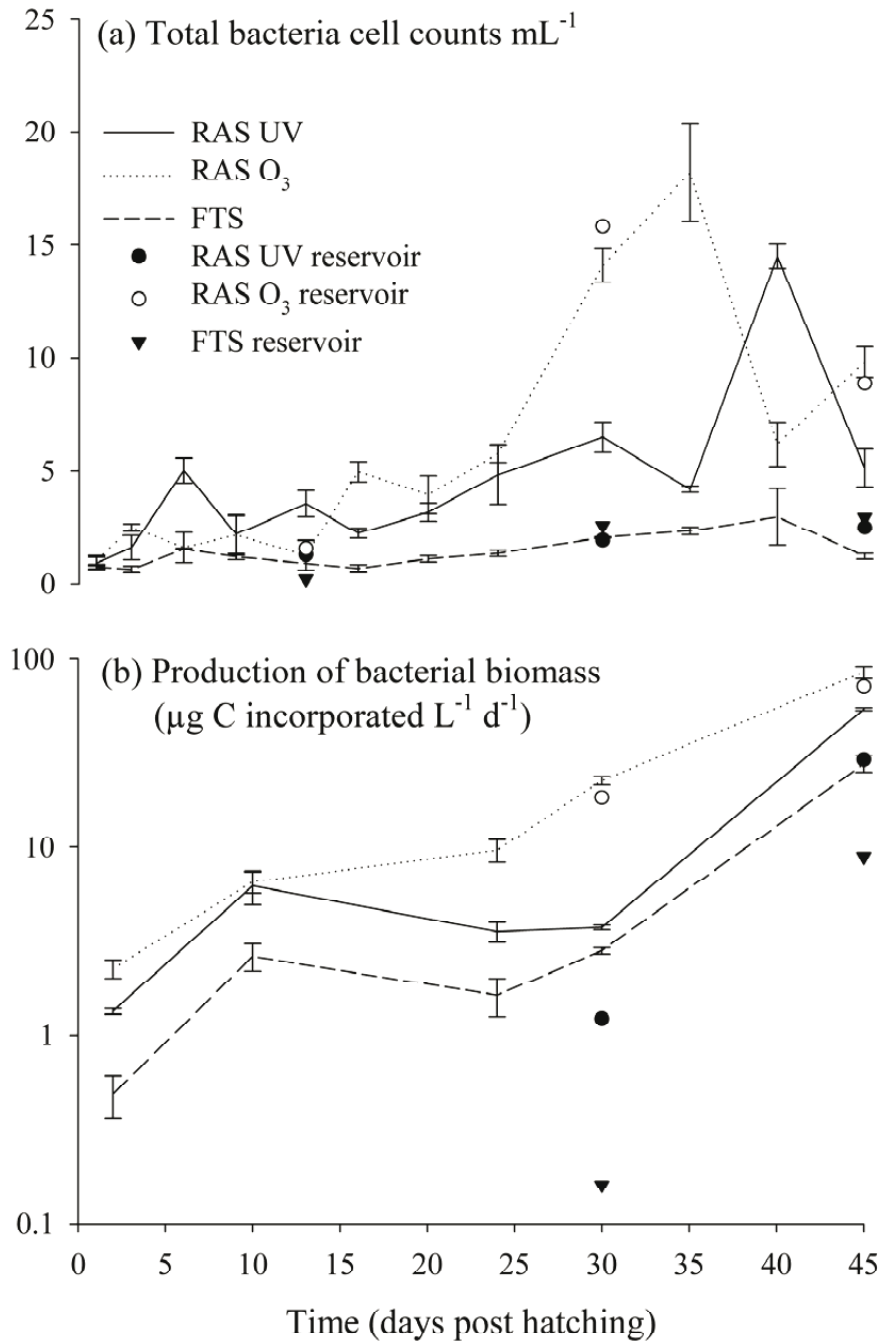
Fig. 2: (a) Total bacteria cell counts and (b) the production of heterotrophic bacterial biomass measured as the amount of leucine used for protein biosynthesis (average ± SE) in the tanks and the water going to the tanks of the RAS UV, RAS O$_3$ and FTS.
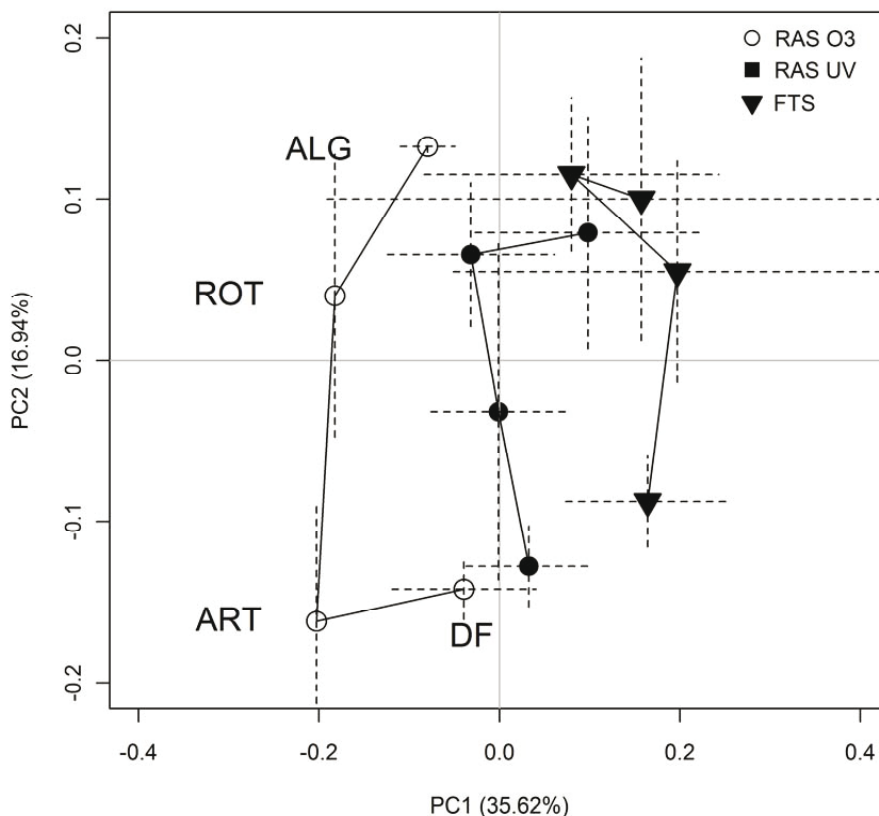
Fig. 3: PCA score plot of T-RFLP data from the three systems with $SD_{PC1} \times SD_{PC2}$ (dashed line) and trajectory lines (solid line). ALG = algae period, ROT = rotifer period, ART = *Artemia* period, DF = dry feed period. The $SD_{PC1} \times SD_{PC2}$ value is directly proportional with the amount of change observed in the T-RFLP fingerprints.

3.3 Performance of fish larvae

Figure 4 shows the change in dry weight of the cod larvae as a function of time and Table 4 shows a summary of the performance of fish in the three systems. The RAS O_3 larvae had significantly higher DW than the larvae in the RAS UV on days 17, 27, 30 and 37 ph ($p < 0.035$), and significantly higher DW than the FTS larvae on days 27, 30, 37 and 45 ph ($p < 0.003$). The FTS larvae showed significantly lower DW than the larvae in the RAS UV at the end of the dry feed period (45 dph, $p < 0.001$).

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One of the replicate RAS O$_3$ tanks stood out with its low survival during the live feed period, whereas the other two replicates showed the highest survival of the experiment. From day 16 ph the density of larvae estimated by photography was significantly higher in the RAS O$_3$ than in the RAS UV and FTS (p = 0.017, Figure 5). Survival was higher in the RAS O$_3$ than in the other treatments through the dry feed period (p = 0.015). The FTS included the tank with the lowest survival of larvae through the experiment.

Table 4: Daily specific growth rate (% SGR) and survival of the fish larvae in the two RAS and the FTS during the live feed and dry feed period (average ± SE).

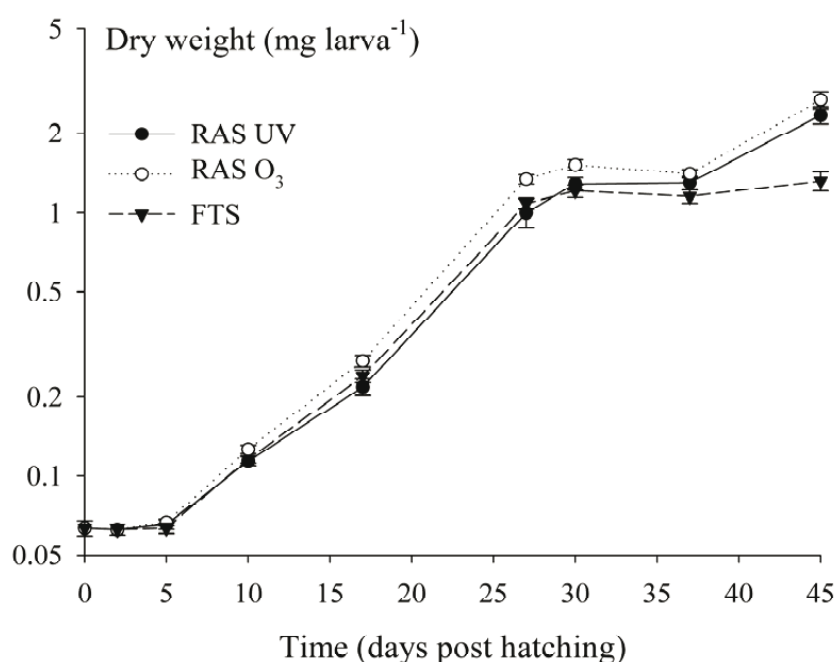| Period (dph)	Live feed (2-30)			Dry feed (31-45)		
System	RAS UV	RAS O$_3$	FTS	RAS UV	RAS O$_3$	FTS
SGR (% d^{-1})	10.1 ± 1.1	10.6 ± 0.9	10.0 ± 1.0	4.0 ± 0.7	3.8 ± 0.7	0.6 ± 0.7
Survival (%)	21.3 ± 1.8	36.0 ± 12.2	16.3 ± 2.0	4.0 ± 0.3	5.4 ± 0.8	1.4 ± 0.8
Survival (%) replicate 1	20.6	54.8	19.1	3.4	4.0	3.0
Survival (%) replicate 2	18.5	13.2	17.5	4.1	5.5	0.9
Survival (%) replicate 3	24.8	40.1	12.4	4.4	6.8	0.3

Fig. 4: Dry weight of the larvae in the three systems (corrected for temperature differences, average ± SE).
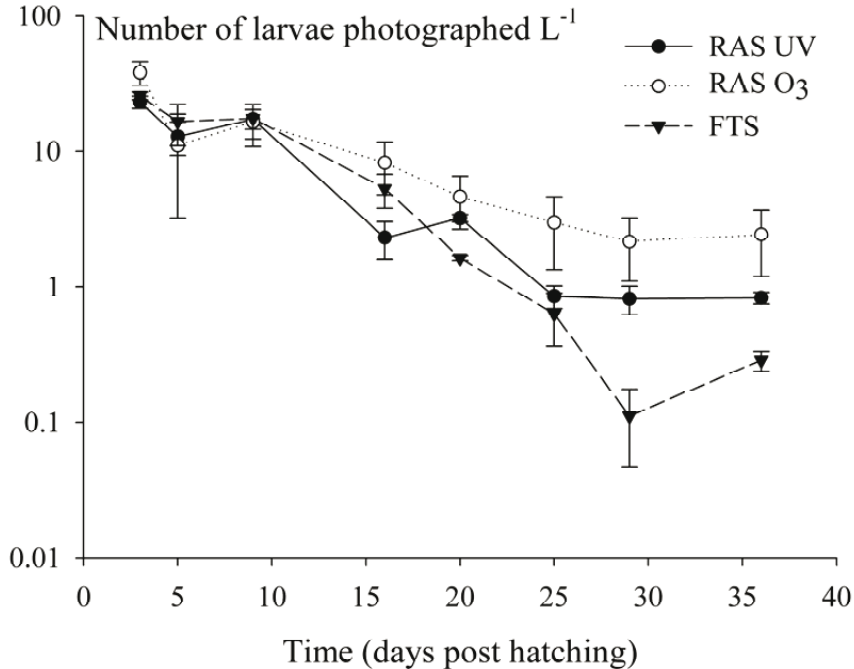
Fig. 5: Density of larvae in the upper water column observed via photography (average ± SE).

4 Discussion

4.1 Microbial community

The microbial community composition of the rearing water developed differently according to water treatment regime. Higher abundance of bacteria, increased production of heterotrophic biomass and elevated concentrations of organic matter (DOC and POC) confirm the expectation that the CC was higher in the rearing water of the two RAS compared to the FTS.

A low CB may suggest a high level of specialised bacteria with a narrow niche width characteristic of a more mature community, and has been used to describe the state of maturation of the microbial community (Skjermo *et al.*, 1997). CB increases with increasing substrate load, and may therefore be higher in microbially matured water (Salvesen *et al.*, 1999) and RAS (Attramadal *et al.*, submitted). In the present experiment, however, CB was higher in the FTS than in the two RAS even if the level of organic matter was about three

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times lower, indicating a less matured community in the FTS. The FTS showed the least stable microbial community composition for each period, and a lower species richness of bacteria, which supports the hypothesis that RAS operation contributes to increased stability of the microbial community in the rearing water (Attramadal *et al*., submitted).

Our results indicate that the type and/or level of disinfection had effects on the ability of the two RAS to mature and stabilise the microbial community of the rearing water. Of the two RAS, the RAS UV resembled the FTS more in microbial composition, abundance of bacteria and microbial activity. The RAS UV showed higher shares of opportunists than the other systems during the *Artemia* period, which indicates a low level of maturation. The RAS O_3 seemed to have the most matured and stable microbial environment of the three systems. The two methods of disinfection may have affected the recolonisation and development of the microbial community differently (Hess-Erga *et al*., 2010), and we suggest that the observed differences in the microbial community were mainly the results of different disinfection efficiencies in the two RAS.

When the treated water and the water in the rearing tanks differ in the level of bacteria and substrate, an imbalance between the resulting CC and the amount of bacteria competing for substrate may be created when the treated water enters the tank. A gap between the concentration of bacteria and substrate in the water going to the tanks and the composition of the microbial community and CC of the water in the rearing tanks is a potential opening for recolonisation and proliferation of opportunists (Salvesen *et al*., 1999, Attramadal *et al*., submitted). In RAS the organic loading, i.e. CC, of the treated water is close to the levels of the rearing tanks. By disinfecting the water in a RAS, however, the amount of bacteria competing for the substrate in the rearing tanks is reduced, destabilising the microbial community and increasing the chances of opportunistic proliferation. The higher stability of the microbial community in the RAS UV compared to the FTS may partly be due to the long water retention time in the RAS. More research is needed to optimise RAS for microbial control in the rearing of marine larvae.

4.2 Physiochemical water quality

Although all three systems had an acceptable physiochemical water quality during the live feed period, the rearing water of the two RAS always contained more nitrogenous waste and organic matter. In addition, the pH decreased in the two RAS from the *Artemia* period

onwards, reflecting higher CO_2 concentration and nitrification. This was comparable with results from other studies (Olivar *et al.*, 2000, Foss *et al.*, 2006, Attramadal *et al.*, submitted). The physiochemical water quality quickly deteriorated during the dry feed period, and this period may be considered a stress test of the larvae. This was partly due to the fact that the pumps were under dimensioned, thus technical adjustment may improve water quality by increasing the water exchange rates of tanks during this period.

RAS depend on bacteria-mediated conversion of ammonia to nitrite and further to nitrate in the biofilter. Both un-ionised ammonia and nitrite are toxic to marine fish larvae and juveniles at low concentrations, with sublethal effects like reduced growth rate (Brownell, 1980, Handy and Poxton, 1993, Parra and Yufera, 1999, Foss *et al.*, 2004, Siikavuopio and Sæther, 2006, Björnsson and Olafsdóttir, 2006). Cod juveniles showed reduced growth rates at concentrations above 0.06 mg NH_3-N L^{-1} (Foss *et al.*, 2004) and at 1.0 mg NO_2-N L^{-1} (Siikavuopio and Sæther, 2006). Levels of un-ionised ammonia and nitrite were considered safe in the two RAS, although the level of nitrite may have been suboptimal for the larvae during the dry feed period (Poxton and Allouse, 1982, Siikavuopio and Sæther, 2006). Especially the RAS UV showed high concentrations of nitrite during the last part of the dry feed period, which may have stressed the larvae. However, cod have also been shown to acclimate to elevated levels of nitrite (Siikavuopio and Sæther, 2006). The ammonia to nitrite conversion step seemed to work satisfyingly in both RAS. However, accumulation of nitrite indicates a slower establishment and response of the nitrite to nitrate conversion step, as typically seen for marine biofilters (Manthe and Malone, 1987). The nitrite oxidation reaction step was not absent, however, as nitrate accumulated.

The accumulation of nitrite and POC were the only physiochemical variables that clearly differed between the two RAS. Ozone oxidation of nitrite to nitrate (Rosenthal and Otte, 1979, Borges *et al.*, 2003) may have contributed to the lower nitrite levels in the RAS O_3 compared to in the RAS UV, but this cannot be concluded from the concentrations of ammonia and the accumulation of nitrate observed in the two systems. The higher POC may indicate a higher turbidity in the RAS O_3 than in the RAS UV and a higher turbidity in the two RAS than in the FTS that may have had an effect on the performance of larvae.

4.3 Performance of fish larvae

In total, fish larvae reared in the two RAS performed at least as well as those reared in the FTS, even if the physiochemical environment appeared to be inferior. This is in accordance with previous results (Attramadal *et al*., submitted). Despite the reduced physiochemical water quality in the two RAS during the dry feed period, the RAS larvae grew faster and survived better than the larvae in the FTS. This is a strong indication that the larvae had obtained a higher viability the first month post hatching that made them more able to cope with the environmental stress they experienced during the dry feed period.

Generally, the performance of larvae could be related to the overall state of the microbial community, the RAS O_3 showing the most favourable development in the microbial environment, including stabilisation and maturation, and the best performance of larvae. The larvae of the RAS UV showed a similar or slightly better performance than the larvae of the FTS. The microbial community of the FTS was the least stable and differed most from the composition of bacteria characterising the RAS O_3.

Little is known about the direct effects of ozonation on the microbial environment or the performance of marine larvae, and one cannot rule out that the ozonation in the RAS O_3 may have influenced the bacteria or the larvae. Significantly different and presumably more beneficial microbial communities in RAS compared to FTS have been described in both rearing water (Verner-Jeffreys *et al.*, 2004) and in larvae (Fjellheim *et al*., 2007, Fjellheim *et al*., submitted). Further knowledge about the functional and taxonomic groups of bacteria associated with the water and the larvae in RAS with moderate ozonation of water could be useful to reveal the mechanisms behind these empirically favourable treatments.

5 Conclusions

Two marine hatchery RAS developed different and more stable microbial community compositions in comparison with a FTS with the same feed and rearing regime. The RAS with moderate ozonation showed a more matured and stable microbial community in comparison with the RAS with UV-irradiation that involved higher disinfection efficiency. Atlantic cod larvae reared in the two RAS performed equally well or better than their FTS siblings, despite exposure to an inferior physiochemical water quality.

Our results support the hypothesis that RAS is applicable as a as a microbial control strategy during the live feed period when the sensitivity of larvae is high, the biomass of the fish is low and the production of waste is limited (Attramadal *et al*., submitted). However, the results indicate that strong disinfection may reduce the microbial maturation and destabilise the microbial community in the RAS, with poorer performance of the larvae as a result. Further research should be conducted to identify which functional groups or species of bacteria are contributing to the observed RAS effect.

The possibility that moderate ozonation may have a beneficial effect on the microbial community development related to the fish or on the larvae directly should be investigated further.

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Paper 3

Ceramic clay reduces the load of organic matter and bacteria in marine fish larval culture tanks

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Abstract

Ceramic clay has been increasingly used to improve contrast and prey detection in tanks for rearing of fish larvae. In contrast to live microalgae or algae pastes, clay increases turbidity without contributing to the organic matter load. In addition, clay may aggregate and sediment organic matter and bacteria, facilitating its removal. Marine larvae are sensitive to infections by opportunistic bacteria. Fish, algae, and live feed increase the microbial carrying capacity of the rearing water which allow exponential growth of bacteria and favour fast-growing opportunists. Reducing substrate levels by replacing microalgae with clay may reduce bacteria proliferation and benefit larvae. We compared the effects of three rearing regimes including live *Isochrysis galbana*, *Nannochloropsis oculata* paste, and ceramic clay on the bacterial community, concentration of organic matter, and growth and survival of Atlantic cod larvae (*Gadus morhua* L.). The application of clay resulted in reduced substrate levels for bacteria in the rearing water compared to the addition of live algae or algae paste. To some extent, clay aggregated and transported organic matter to the bottom of the larval fish tanks, where it could be effectively removed. Fish tanks receiving clay showed a lower abundance of bacteria in the water than tanks added algae paste or live algae. Fish tanks with algae paste showed a higher abundance of bacteria and a higher share of cultivable bacteria and TCBS counts than the other two treatments. Tanks with live algae showed low relative abundances of opportunistic bacteria and TCBS counts in both water and rotifers. Cod larvae in tanks with clay or live algae initiated exponential growth earlier than larvae in tanks with algae paste. Larvae in tanks receiving clay had significantly higher dry weight than larvae in tanks receiving algae paste at day 5 and 20 post hatching. Two of the three tanks with clay had significantly higher larval survival than the tanks with live algae or algae paste. However, one tank with clay underwent 100 % mortality. Clay addition appears to be an easy way to reduce bacterial load during early first feeding of marine larvae without compromising the beneficial effects of turbidity.

1 Introduction

Production of juveniles is a bottleneck in marine aquaculture, characterized by variable performance of larvae, which has been linked to negative interactions with microbes (Vadstein *et al.*, 1993, 2004). Newly hatched marine larvae rely on the general immune system and are vulnerable to infections by opportunistic bacteria. Non-selective reduction of bacteria is one of three key elements suggested in a strategy aiming for microbial control in the rearing of marine larvae (Vadstein *et al.*, 1993). According to this strategy, actions aimed at limiting the abundance of bacteria include methods that focus on reducing the microbial carrying capacity (CC) of the system by reducing input and increasing removal of organic matter. Dissolved organic matter (DOM) supplied from decomposing hatching remnants, fecal matter, and live feed is the main growth-limiting substrate for heterotrophic bacteria in rearing water. Different types of particles are commonly added to the rearing water during the first feeding of marine larvae. We hypothesize that the addition of ceramic clay reduces the load of organic matter on the rearing tanks, and hence the abundance of bacteria, compared to addition of algae paste or live microalgae.

Addition of microalgae to the culture water has a beneficial influence on survival and growth of marine larvae in intensive rearing systems (Howell, 1979, Naas *et al.*, 1992, Reitan *et al*., 1993, Salvesen *et al*., 1999, Lazo *et al*., 2000). This "green water" effect has been attributed to turbidity which improves foraging conditions by affecting prey contrast and larval distribution (Naas *et al.*, 1992). Microalgae may also have a beneficial nutritional impact as feed for live prey and larvae (Reitan *et al.*, 1993, 1994, 1997), and may stimulate digestive enzyme activity (Cahu *et al*., 1998). Moreover, addition of live microalgae has been shown to have a positive effect on the microbial environment of the culture water (Skjermo and Vadstein 1993, Salvesen *et al.*, 1999) and may accelerate the initial bacterial colonisation of the fish gut (Bergh *et al*., 1994).

Other sources of turbidity, like commercially available microalgae pastes and ceramic clay, are used in many hatcheries because they are labour- and space saving compared to live algae. Compared to clear water, the addition of clay to culture tanks has been shown to increase ingestion rates and improve performance of larvae of Atlantic halibut (*Hippoglossus hippoglossus*) (Naas *et al*., 1995) and walleye (*Stizostedion vitreum*) (Bristow and Summerfelt, 1994, Bristow *et al*., 1996, Rieger and Summerfelt, 1997), as well as Pacific

oyster (*Crassostrea gigas*) (Matson *et al*., 2006). Clay is increasingly used in the first feeding of halibut (Harboe and Reitan, 2005, Björnsdóttir, 2010), and is more cost efficient than the application of either live algae or algae paste.

The different particles used to condition rearing water (live algae, algae pastes, and clay) have different surface properties and represent different levels of contribution of organic matter and associated bacteria. Living microalgae release organic matter (Baines and Pace, 1991) and senescent and decaying phytoplankton serve as bacterial substrate (Cole *et al*., 1984). Live algae cultures contain algae cells, associated bacteria and algal metabolites, sometimes including antibacterial compounds. Addition of live microalgae represents a daily supply of DOM and bacteria to the rearing water, and may influence the bacterial community of the larval rearing tanks (Skjermo and Vadstein, 1993, Salvesen *et al*., 1999). Algae paste is a mixture of weakened cells and cell remnants, which may include active algal metabolites. Algae may serve as food for live feed and larvae (Reitan *et al.*, 1993, 1994, 1997).

In contrast to microalgae, clay contributes little to the DOM or microbial load to the fish tanks. Consequently, the supply of bacterial substrate can be reduced by substituting microalgae with clay to condition the rearing water. Moreover, application of clay may contribute to direct removal of organic matter and bacteria from the fish tanks by adsorption and precipitation. Clay may bind and aggregate organic matter in fresh water (Lind *et al*., 1997, Tietjen *et al*., 2005), estuarine water (Landau *et al*., 2002) and sea water (Satterberg *et al*., 2003), and is commonly used as a fining agent to precipitate and remove suspended organic compounds in wine and juice production (Zoecklein, 1988, Blade and Boulton, 1988). Bacteria cells in the rearing water may adhere to clay or aggregates of clay and organic matter (Shchur *et al*., 2004). Bacterial adhesion to a colloid particle is complex, depending on the electric double layer and van der Waal forces as described in the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloid stability (Hermansson, 1999). The ionic strength influences bacterial adhesion by affecting the electric double layer. Factors like characteristics of the bacterial surface, cell wall hydrophobicity, and motility also affect adhesion (Van Loosdrecht *et al*., 1987, Huysman and Verstraete, 1993, De Kerchove and Elimelech, 2008, De Schryver *et al.*, 2008). Precipitated aggregates and settled clay can easily be removed from the bottom of fish tanks. Alternatively, Landau *et al*. (2002) showed that clay with adsorbed protein may be removed by foam fractionation.

Three experiments were conducted to compare the effects of live microalgae, algae paste, and clay on the concentration of DOM and microbial conditions in rearing of Atlantic cod (*Gadus morhua* L.). Experiment 1 was a preliminary study carried out to investigate if clay aggregates and transports organic matter from the water column to the bottom of fish tanks more efficiently than live microalgae or algae paste. As most of the information on clay addition in aquaculture is from halibut production, little is known about its effect on rotifers. Experiment 2 was set up to study how clay addition influences rotifer concentration and the microbial composition of water and rotifers in the rearing tanks in the absence of fish. Experiment 3 was carried out to study how clay addition affects growth and survival of cod larvae, the concentration of organic matter, and the development of the microbial community of the water in comparison with live algae or algae paste.

2 Materials and methods

2.1 Experimental setup

Live *Isochrysis galbana* (LA) cultured in semi-continuous culture, algae paste of *Nannochloropsis oculata* (AP) (Instant Algae, Reed Mariculture, California) and ceramic clay (CL) (Vingerling K148, WBB Fuchs GmbH, Germany) were applied in three experiments. According to the provider, the ceramic clay had been ground (< 200 µm) from raw materials like quartz, feldspar, ballclay and clay, the main clay mineral being an illitic mineral. The dose of clay added was chosen on the basis of the resulting turbidity in tanks (~10 cm Secchi depth in Experiment 2 and 3). The structure of the clay was visualized from uncoated dried samples in field emission microscopes (Zeiss Supra 55VP and Zeiss Ultra 55) at the NTNU Department of Materials Science and Engineering. Sea water was pumped from 60 m depth in Trondhjemsfjorden (pH 8.0, 34 ppt salinity) and sand filtered. Batch cultured rotifers (*Brachionus* 'Nevada'), fed *N. oculata* algae paste, Baker's yeast, and Marol-E (rotifer oil emulsion enrichment, SINTEF, Trondheim), were used in Experiment 2 and 3.

2.1.1 Experiment 1

Experiment 1 was a preliminary test carried out in four black polyethylene tanks (70 L, flat bottoms, central aeration). Water exchange rate was 2 tank volumes d^{-1}. Temperature was 12°C. One tank received live algae (2 mg C L^{-1} final concentration), a second algae paste (2 mg C L^{-1} final concentration), and a third tank was added clay (100 mg L^{-1} final

concentration). After 30 min, these three tanks, along with a fourth tank (Control) received 25 mg L^{-1} yeast extract (Oxoid, UK) to simulate increased organic substrate levels characteristic at several points during first feeding (e.g., hatching, feeding). Samples of settled material were collected in two open Petri dishes placed on the bottom of each tank before addition of particles. Each dish was held in place by two clean stainless steel nuts. Settled material was sampled after 24 h by slowly placing lids on the Petri dishes and lifting them out of the tanks. Samples were analysed for dissolved and particulate organic carbon (DOC and POC, respectively) concentration, and normalized to sedimented material per volume of rearing water.

2.1.2 Experiment 2

Experiment 2 was carried out in nine black polyethylene tanks (160 L, coned bottoms) with central aeration for 7 days at 12°C. Water exchange rate was 1 tank volume d^{-1}. Tank bottom debris was removed every other day by siphoning. Rotifers were distributed (~3 mL^{-1}) to the tanks two times each day to simulate feeding. Three tanks received live algae (2 mg C L^{-1} final concentration), three tanks algae paste (2 mg C L^{-1} final concentration), and three tanks received clay (30 mg L^{-1} final concentration) at the same time as the rotifers. Rotifer density was estimated continuously using a rotifer counter (Alver *et al*., 2007), and counted manually once a day. Tank water and rotifers were analysed for bacteria after 3 and 6 days.

2.1.3 Experiment 3

Experiment 3 was a first feeding experiment, and Table 1 summarizes the rearing regime used. Atlantic cod eggs (63°d) were received from Havlandet Marin Yngel AS. The eggs were disinfected in 400 ppm glutaraldehyde for 6 minutes upon arrival (Salvesen and Vadstein, 1995), and incubated at 7.5°C in hatching incubators to 85°d, when they were transferred to nine 160 L black, coned circular polyethylene fish tanks at a final density of 100 individuals L^{-1}. The hatching success was 99 %.

Larvae were hatched and maintained in darkness the first 3 days and then exposed to continuous light. Tank outlets were central perforated pipes covered with nylon net (400 μm). The tanks had gentle aeration at the bottom, and were equipped with surface skimmers to collect wastes gathering in the surface film. Water exchange rates were gradually increased from 1 to 6 tank volumes d^{-1}. Temperature was increased gradually from 7 to 12°C during the experiment. Debris was removed from the bottom of the tanks every other day. After, starting

on day 3 post hatching (ph), rotifers were distributed to the fish tanks three times each day to obtain a final concentration of ~5 individuals mL^{-1}. Live microalgae and algae paste was added to three tanks each at the times of feeding (1.5 mg C L^{-1}). Ceramic clay (30 mg L^{-1}) was added to three additional tanks two times each day in connection with feeding. The rearing water was sampled to measure the concentration of organic matter (DOC and POC) and bacteria. Water was sampled before feeding and was prefiltered (50 µm) to exclude rotifers. The total number of fish surviving to 20 days post hatching (dph) was counted after termination with an overdose of Tricaine Methanesulfonate (MS222).

Table 1: Rearing regime, Experiment 3.

Total management	Days post hatching
	0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Water exchange (tank volume d^{-1})	1 ⟶ 2 ⟶ 3 ⟶ 4 ⟶ 6 ⟶
Light	Dark Continuous light ⟶
Feed	Rotifers ⟶
CL-treatment	Clay ⟶
LA-treatement	Live algae (*Isochrysis galbana*) ⟶
AP-treatment	Algae paste (*Nannochloropsis oculata*) ⟶
Temperature (ºC)	7 8 9 10 11 12 ⟶

2.2 Analytical procedures

2.2.1 Organic matter and turbidity

DOC and POC samples were immediately vacuum filtered through ignited (480ºC, 2 h) 0.7µm, 25mm diameter GF/F glass microfiber filters (Whatman International Ltd., England). Filtrate and filters were stored at -20ºC. Filtrate was analysed for DOC in a Tekmar-Dohrmann Apollo 9000 TOC-analysator (Teledyne Tekmar, USA). Inorganic CO_2 was removed from filters in a hydrochloric acid saturated atmosphere (37 %, 20 min). Each filter was transferred to a tin cup (Säntis Analytical AG, Switzerland) and analyzed for POC in a CHN Elemental Analyser 1106 (Carlo Erba Instruments, Italy). Turbidity was determined by optical density, OD, at 750 nm on a Shimadzu double-beam spectrophotometer (UV-150-02).

2.2.2 Colony forming units

Rotifers were concentrated on a 50µm sieve, rinsed in autoclaved sea water, and homogenized (~500 individuals mL^{-1}). The number of colony forming units (CFU) was determined in the rotifer homogenate and water samples. Two agar types were used: M-65 seawater agar (Skjermo and Vadstein, 1999) and TCBS agar (Difco, BD Diagnostic Systems, USA). Three

10-fold dilutions were plated from each sample, and each dilution was plated in triplicate. Samples were incubated in darkness at $12 \pm 1°C$. Plates containing 30-300 colonies were preferably counted. Total CFU was calculated as the average of colonies on triplicate M65 plates after 14 days of incubation. Because r-strategic opportunists are characterised by high maximum growth rates, in contrast to K-strategic specialists, the fraction of fast-growing bacteria of total CFU may be used as a measure of the relative presence of opportunistic bacteria (Skjermo *et al.*, 1997, Salvesen and Vadstein, 2000). In this paper, the term opportunistic bacteria is used to denote the CFU emerging the first two days of incubation on M65 agar as described by Salvesen and Vadstein (2000). Visible colonies on TCBS plates were also counted after 2 days of incubation, thus the TCBS counts represent the opportunists that grow on this agar type. Several *Vibrio* species form colonies on TCBS agar (Randrianarivelo *et al*., 2010), but TCBS counts may also represent bacteria from other taxonomic groups (Lopez-Torres and Lizarraga-Partida, 2001).

2.2.3 Total bacteria cell numbers

Total bacterial cell numbers (total cell counts) were determined by fluorescence microscopy (Hobbie *et al*., 1977). Samples were fixed with glutaraldehyde (1 % final concentration) and stored dark at 4ºC. Two mL of sample was diluted with 3 mL milli-Q water and vacuum filtered onto black polycarbonate filters (0.22μm 25mm diameter, Poretics Corp., USA) on supporting mixed cellulose ester membrane filters (0.45μm 25mm diameter, Whatman, UK). Three mL of DAPI (4.6-diamidino-2-phenylindole, 1 mg L^{-1} dH_2O) was added to stain the bacteria for 10 min (Porter and Feig, 1980). The dye was removed by filtration and the filters stored dark and dry. Stained bacteria were counted in an epi-fluorescence microscope (Axioplan 2, Zeiss, Germany) at 1250× magnification using UV excitation. A minimum of 250 individual bacteria, in at least 5 different random squares on the filter was counted for each sample. The fraction of cultivable bacteria (CB) was calculated as the total CFU divided by total cell counts.

2.2.4 Cod larval growth

Larvae sampled for carbon biomass analysis (n = 12-20) were sacrificed with an overdose of MS222, rinsed in fresh water and transferred into individual tin cups (Mikro Kemi AB, Sweden), dried (60ºC, 48 h), and analyzed in a CHN Elemental Analyser 1106 (Carlo Erba Instruments, Italy) at 1020ºC. Carbon and nitrogen contents were quantified chromatographically using standard curves obtained by analyzing acetanilide (C_6H_9NO).

Individual dry weight (DW) of larvae was calculated from measured carbon content using a conversion factor of 2.34 (Reitan *et al.*, 1993). Daily percentage specific growth rate (% SGR) was calculated from larval dry weight (DW) at time t according to Equation 1 and 2:

$$SGR\ (d^{-1}) = (\ln DW_t - \ln DW_0)\ /\ (t\text{-}t_0) \tag{1}$$

$$\%\ SGR\ (d^{-1}) = (e^{SGR-1}) \times 100\ \% \tag{2}$$

2.3 Statistical analyses

Mean ± standard error of the mean (SE) is presented. Statistical analysis was performed at the 95 % confidence level (p < 0.05). Data for turbidity, rotifer density and microbiology was tested for differences between treatments with one-way ANOVA and post hoc Tukey B in SPSS (SPSS16.0, SPSS Inc., USA). Data for larval dry weight were tested for differences between tanks within each treatment and between treatments for each sample day by Kruskal–Wallis one-way analysis of variance and Mann-Whitney U tests in SPSS. Non-parametric tests were used as the variance in the dataset was unstable even after log transformation. For % SGR, SE was calculated from linear regressions of log transformed individual DW data (Sigmaplot, Systat Software Inc., USA). Because only two of the three replicate CL tanks remained on day 20 ph, the SGR was not statistically tested.

3 Results

The ceramic clay used consisted of leaf like particles less than 1 μm in size (Figure 1), thus giving the clay a high surface area to weight ratio.
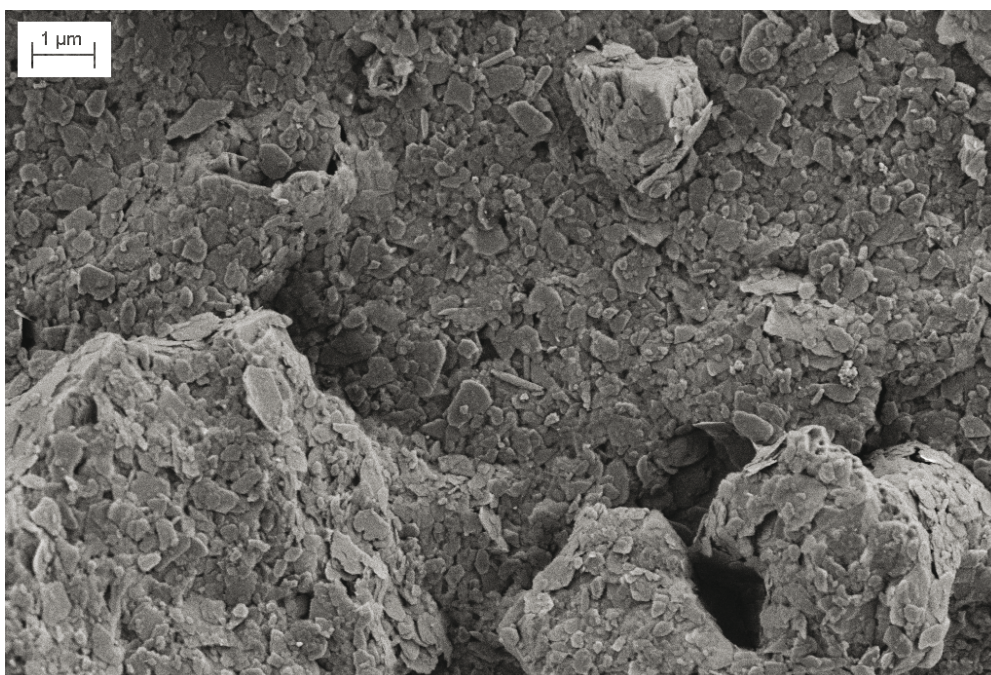

Fig. 1: Field emission microscopy picture of the ceramic clay (photo by Tor A. Nilsen).

3.1 Experiment 1

More sedimented organic matter was found on the bottom of CL tanks than in the LA or AP tanks 24 h after substrate addition (Figure 2). This was mainly due to a higher content of particulate organic matter. The clay was inorganic, which means it represented particles but was not registered as POC unless associated with organic matter. No algal cells or other forms of POC were added to the tanks with the inorganic clay; hence POC on the bottom of CL tanks represented aggregates of clay and organic matter. The particulate organic matter on the bottom of LA and AP tanks was mainly algae, as observed via microscopy. Thus, the results presented in Figure 2, is an underestimation of the ability of clay to adsorb DOM.

The volume of the layer including debris on the bottom of the tanks was relatively small compared to the total volume of the tank. This was reflected in the fraction of waste that could

be removed from the total volume of the tank during cleaning: 1 cm of bottom layer (2 L) corresponded to 3 % of the total tank volume. Total organic carbon concentration (DOC + POC) was almost double in the bottom layer of the CL tank (9 mg L^{-1}) compared to in the water column (5 mg L^{-1}) after 24 h, which meant that about 5 % of the total organic matter in the tank could be removed with the sedimented clay during cleaning. Intake water contained 2 mg C L^{-1}.
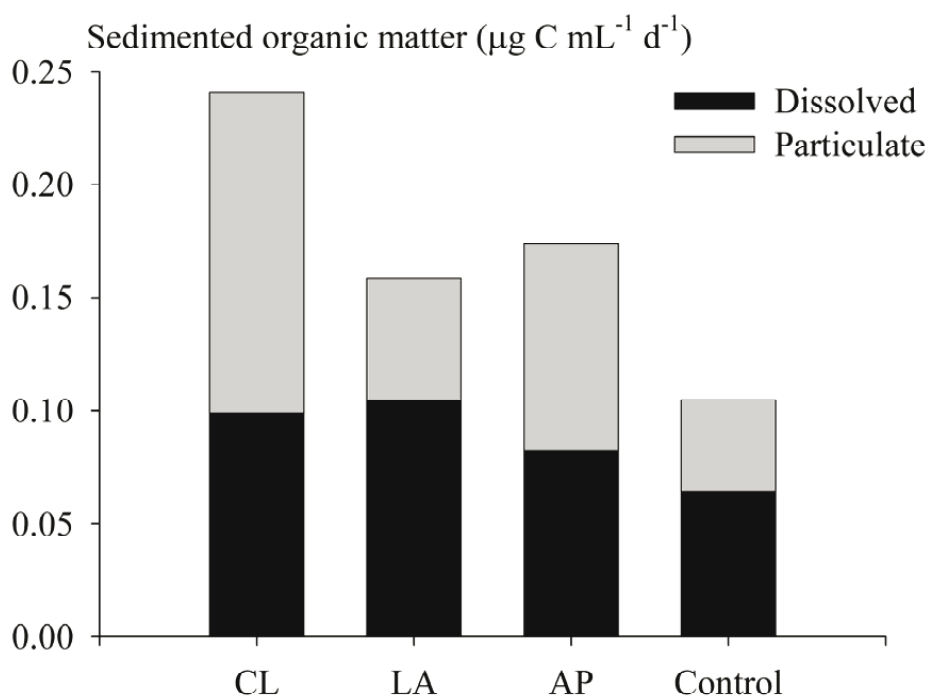

Fig. 2: The amount of dissolved (black) and particulate (grey) organic matter sedimented to the 1 cm of bottom water per mL of rearing water per day in tanks with yeast extract (Control) and clay, live algae or algae paste (Experiment 1).

3.2 Experiment 2

In Experiment 2, the turbidity (OD at 750 nm) of the culture water was in the same range for all treatments, but higher in tanks with clay (p < 0.001, Figure 3). Rotifer densities were in the same range and the pattern of variation was similar for all treatments for one week when no fish were present (Levene's test for equality of variances, Figure 4). However, on average over the experiment, the CL tanks tended to have lower densities of rotifers than the AP tanks

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(p = 0.035, manual counts, Figure 4 a). AP tanks showed a higher density of rotifers than the two other treatments on average for the period with automatic rotifer counts (p < 0.001, Figure 4 b). It should be noted that the observed trend may unintendedly originate from other sources than particle addition, for example the feeding procedure.
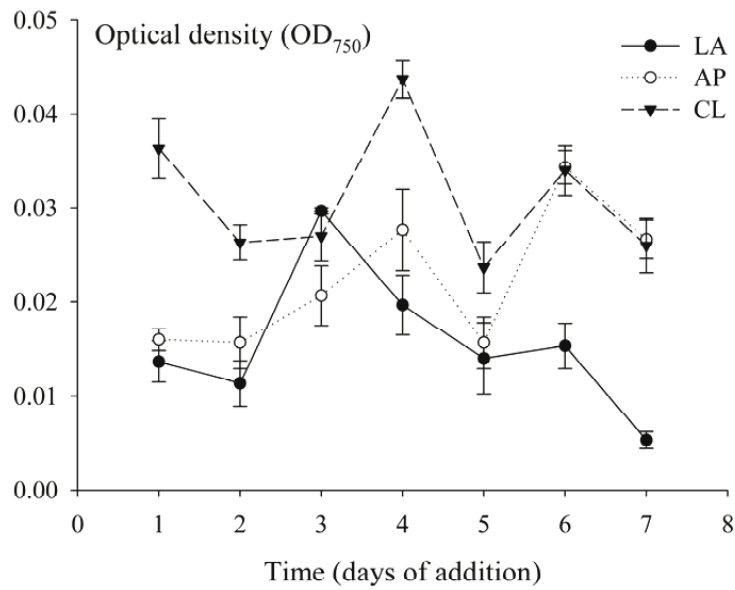

Fig. 3: Absorbance (OD_{750}) in tanks receiving live algae (●), algae paste (○), and clay (▼) for one week (Experiment 2).
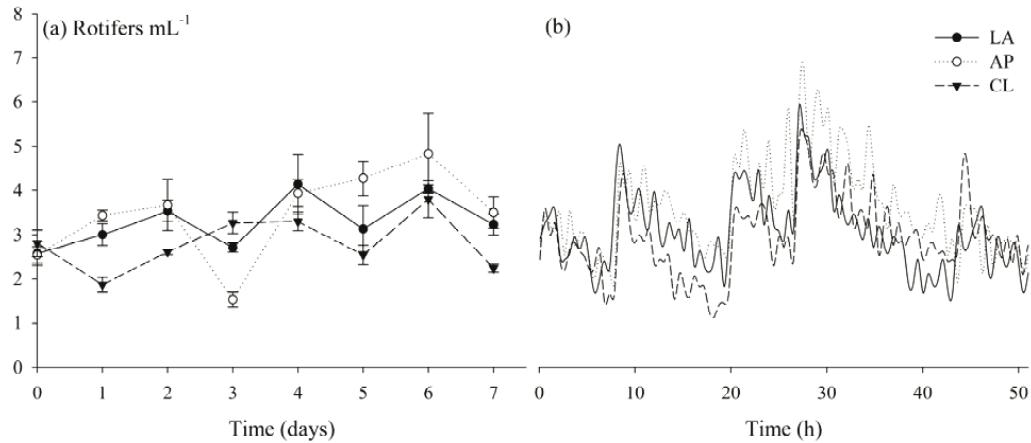
Fig. 4: Rotifer density (a) counted manually for 6 days and (b) counted automatically for 50 hours, in the absence of fish in tanks receiving live algae (solid line, ●), algae paste (dotted line, ○), or clay (dashed line, ▼) in Experiment 2.

In Experiment 2, which did not involve fish larvae, AP tanks showed a higher density of colony forming bacteria in the water than the CL tanks ($p = 0.024$, Figure 5 a). CFU per rotifer was similar in all treatments (Figure 5 a). The LA tanks showed low proportions of TCBS counts and opportunistic bacteria in water and rotifers (except on day 6) compared to the other treatments (Figure 5 b and c). AP tanks showed a higher fraction of TCBS counts in the water than the other two treatments ($p = 0.017$, Figure c), and both the AP and CL tanks showed relatively high fraction of TCBS counts and opportunists in the rotifers after three days. Generally, a higher fraction of opportunists and TCBS counts were present after three days of operation than after six days.
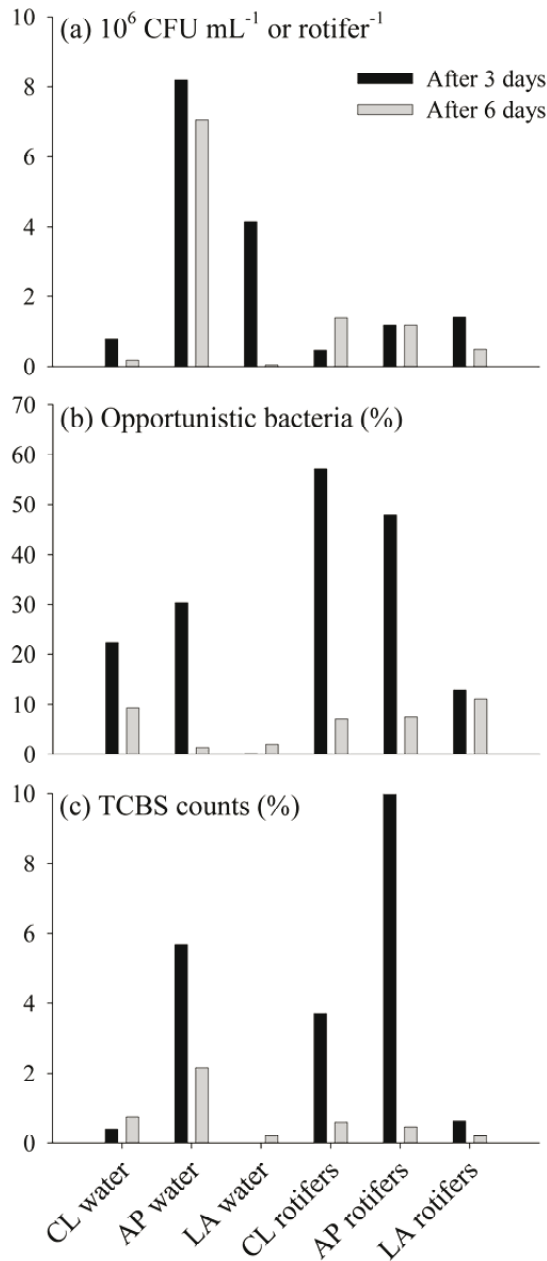
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Fig. 5: (a) Colony forming bacteria, (b) the fraction of opportunistic bacteria and (c) the fraction of TCBS counts of total CFU in water and rotifers of tanks receiving clay, live microalgae, or algae paste in the absence of fish after 3 (black) and 6 (grey) days of particle addition (Experiment 2).

3.3 Experiment 3

In the first feeding experiment, the concentration of DOC and POC increased as a result of the addition of algae (Figure 6). Both components remained stable at a comparable level to the intake water in the CL tanks, and significantly lower than in the other treatments ($p < 0.001$). In tanks receiving algae, the stable elevated level of organic matter (the sum of DOC and POC: ~4 mg C L^{-1}) reflected the concentration of algae added (1.5 mg C L^{-1}) in addition to the background level of the intake water (2 mg C L^{-1}). The POC concentration was similar in tanks receiving live algae and algae paste; whereas the DOC concentration was slightly higher in the LA tanks compared to the AP tanks ($p < 0.001$).
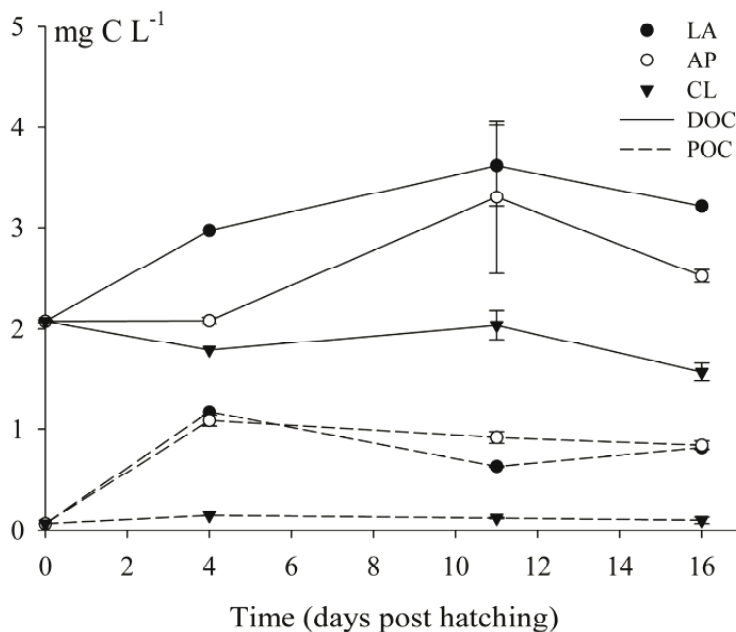
Fig. 6: Dissolved organic carbon, DOC (solid line), and particulate organic carbon, POC (dashed line), in fish tanks receiving live algae (●), algae paste (○), and clay (▼) in Experiment 3.

The abundance of bacteria was higher and more variable in the AP tanks than in tanks of the other two treatments ($p = 0.001$ for CFU and total counts, Figure 7 a and b). The CL tanks showed significantly lower total bacteria counts than the other two treatments ($p < 0.001$). The fraction of cultivable bacteria was generally low (< 5 %), except in the AP tanks, which showed 6 %, 25 % and 51 % CB on day 3, 8 and 16 ph, respectively. The LA tanks showed

lower fractions of opportunistic bacteria in the water the first 8 dph (p = 0.002, one extreme value in one replicate AP tank excepted, Figure 7 c). AP tanks showed higher TCBS counts than the tanks of the other treatments through Experiment 3 (p = 0.022). One of the replicate tanks receiving clay had a markedly higher TCBS counts on day 3 ph (5×10^3 mL^{-1}), which was similar to the level of TCBS counts found in the AP tanks. If that one TCBS count from the CL tank is left out, AP tanks also showed a higher fraction of TCBS counts of total CFU than the tanks of the other treatments through Experiment 3 (p = 0.019, Figure 7 d).

The CL tank with a particularly high TCBS count at 3 dph had total larvae mortality on day 13 ph. In the remaining two CL tanks, fish larvae showed a higher survival than in any tank of the other two treatments (Figure 8 a). AP tanks had higher survival than LA tanks (p = 0.005, t-test). The relatively low survival in LA tanks was probably due to fast-growing filamentous bacteria (the species was not determined) that overgrew these tanks the last week of the experiment. Many seemingly healthy larvae were observed trapped and dead in the bacteria filaments. Filaments of bacteria were also observed in the other treatments, but to a smaller extent compared to that in the LA tanks.
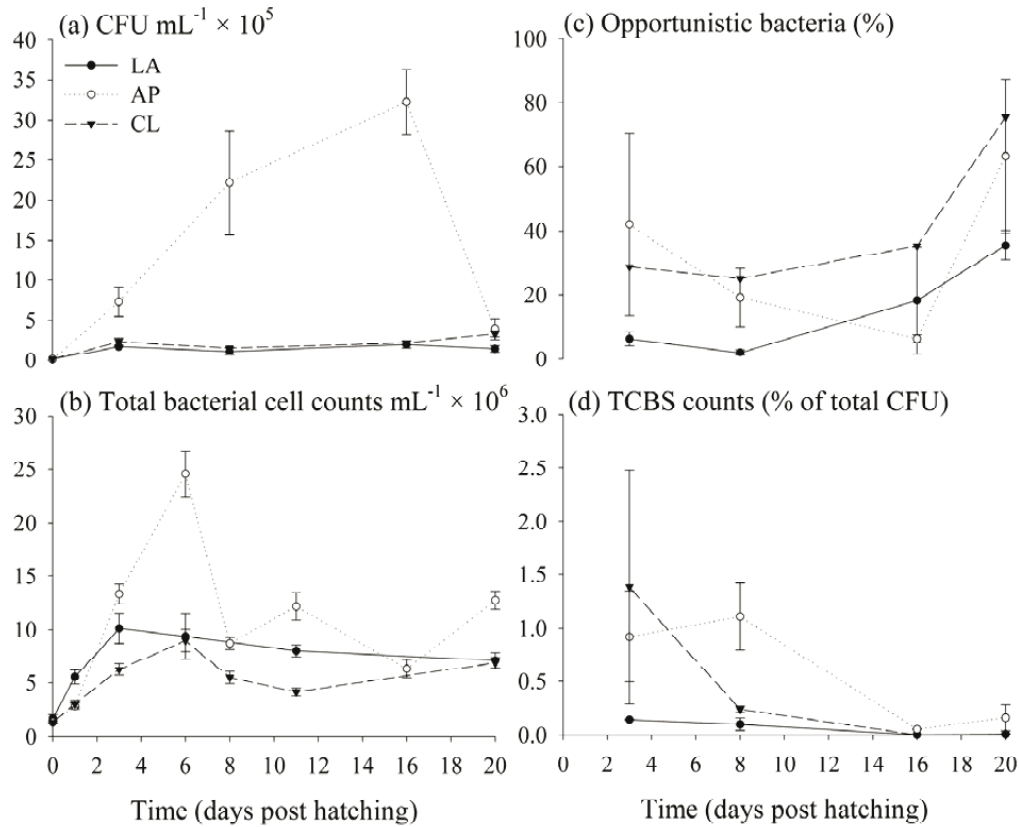
Fig. 7: (a) Colony forming units, CFU, (b) total bacterial cell numbers, (c) the fraction of opportunistic bacteria and (d) the fraction of TCBS counts of total CFU in the fish tanks receiving live algae (●), algae paste (○), and clay (▼) in Experiment 3.

Cod larvae in the CL and LA tanks initiated exponential growth earlier than AP larvae (Figure 8 b). The variation in dry weight among replicate tanks, as well as among individual larvae, was higher for the LA treatment. Significant differences in larval DW were found between replicate tanks with live algae at 5 and 20 dph (p = 0.035 and p = 0.043, respectively), whereas no such difference was found in the other treatments. The coefficients of variation (CV), representing differences among individuals within each treatment, were 27 %, 31 %, and 33 % for the DW of CL, AP and LA larvae, respectively, on day 20 ph. CL larvae had significantly higher DW than AP larvae at 5 and 20 dph (p = 0.016 and 0.025, respectively). The daily percentage specific growth rates (0-20 dph) are shown in Table 2.

Fig. 8: (a) Survival and (b) growth of cod larvae reared in water receiving live algae (●), algae paste (○), and clay (▼) in Experiment 3. Significant difference is marked with an asterisk in (b).

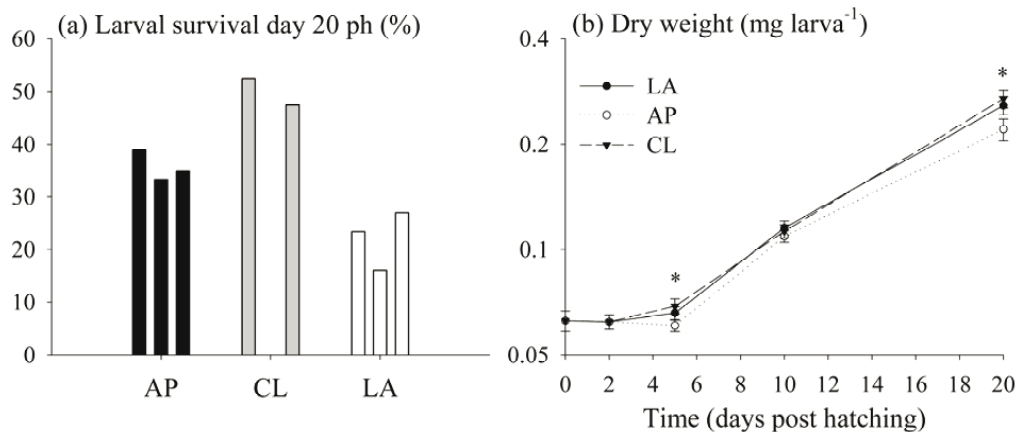**Table 2:** Daily percentage specific growth rates of cod larvae from 0 to 20 dph in tanks receiving live algae, algae paste, or clay (Experiment 3).

% SGR (0-20 dph)	LA	AP	CL
Tank 1	8.0 ± 0.7	6.2 ± 0.5	7.5 ± 0.5
Tank 2	6.2 ± 0.6	6.4 ± 0.7	7.1 ± 0.7
Tank 3	6.8 ± 0.6		
Total	7.1 ± 0.5	6.3 ± 0.5	7.3 ± 0.5

4 Discussion

Clay may, like microalgae, be used to improve light conditions for foraging compared to clear water (Bristow and Summerfelt, 1994, Bristow *et al*., 1996, Rieger and Summerfelt, 1997). The use of clay is cost effective compared to the use of live algae or algae pastes. Substitution of microalgae with inorganic clay appeared to be an effective method to reduce the concentration of organic matter and bacterial proliferation during first feeding of marine larvae without compromising the optimal turbidity. Rotifers and fish larvae did not seem to be adversely affected by the clay in our experiments. On the contrary, the performance of larvae in tanks with clay was good. Table 3 shows a comparison of the organic matter concentration, microbial environment, and fish performance among tanks with live microalgae, algae paste, or inorganic clay (Experiment 3).

Table 3: Comparison of the organic matter concentration, microbial environment, and fish performance for tanks receiving live algae (LA), algae paste (AP), and clay (CL) in Experiment 3.

Treatment comparison	LA	AP	CL
Dissolved organic carbon	High	High	Low
Particulate organic carbon	High	High	Low
Bacterial abundance	Low	High	Low
Fraction of opportunistic bacteria	Low	Moderate	Moderate
Fraction of cultivable bacteria	Low	High	Low
Larval growth	High	Low	High
Larval survival	Low	Moderate	High[a]

[a]Except from one tank which had total mortality 13 dph

4.1 Effects on the concentration of organic matter

The concentration of total organic matter, which includes cells of microalgae, remained low in tanks with inorganic clay compared to tanks with live *I. galbana* or paste of *N. oculata*. Microalgae represent a source of both POC and DOC to the rearing tanks, as DOC may be released from lysed algae cells and is excreted from healthy living microalgae (Brock and Clyne, 1984). We supplied rearing tanks with POC in the form of live algae or algae paste at concentrations of 1.5-2 mg C mL^{-1} and DOC at concentrations comparable to that (Olsen *et al.,* 2002), although not quantified. This resulted in about 2 mg C L^{-1} increase in the total organic carbon concentration (evenly apportioned between DOC and POC) compared to that in tanks conditioned with the inorganic particles of clay (Figure 6).

A rearing regime with clay may reduce the concentration of organic matter compared to that for "green water" protocols in two ways: 1. by reducing the input and 2. by increasing the rate of removal. The presented experiments were designed to compare the effects of rearing regimes including addition of clay or microalgae, and further research is necessary to fully elucidate the relative importance of the two different processes. However, our results allow for some rough estimates of the magnitude of their impact. Each addition of microalgae increased the organic carbon concentration by about 2 mg C L^{-1}. Three additions in 24 hours corresponds to an average increase in the total organic carbon concentration of 70-150% for water exchange rates of 4-1 d^{-1}, respectively, and a concentration of 2 mg C L^{-1} in the intake water. Considering this, we suggest that the input of organic matter was approximately halved

by replacing algae with clay. The ceramic clay bound and sedimented organic matter from the culture water, facilitating removal of bacterial substrate. However, even if clay doubled the total organic matter concentration on the tank bottom compared to that in the rearing water, removal of debris only amounted to about 5 % per day of the total organic matter in the tanks. The greatest effect of using clay instead of algae on the organic matter concentration resulted from a lower input of organic matter, whereas the removal through sedimentation played a minor role.

Most of the increase in the concentration of small suspended organic particles (< 50µm) and DOM in the tanks could be attributed to the addition of algae. This indicates that the amount of POC and DOC supplied due to hatching, fish defecation, and from rotifers was moderate in comparison. Aggregated microbes and a great part of the free-living bacteria cells are likely to be retained on the GF/F filters and included in the POC category (Lee and Fuhrman, 1987, Olsen *et al.*, 2002). The average bacterial carbon concentration in Experiment 3 was 4 µg C L^{-1} in the CL tanks and 17 and 26 µg C L^{-1} in the LA and AP tanks, respectively, assuming a carbon biomass of 20 fg C bacteria cell^{-1} (Lee and Fuhrman, 1987).

4.2 Effects on the microbial community

Water treatment and production routines may destabilise the microbial environment in marine larval rearing. Disinfection of intake water reduces microbial numbers and thus the competition for substrate. Addition of algae, hatching and defecation by live feed, and fish increase the organic matter concentration and the microbial carrying capacity (CC) in larval tanks compared to the intake water (Vadstein *et al.*, 1993, 2004). This favours rapid growth to the higher CC and can result in proliferation of opportunistic heterotrophic bacteria during the first days of cultivation (Skjermo *et al.*, 1997, Salvesen *et al.*, 1999).

The abundance of bacteria was markedly higher in the AP tanks than in tanks of the other two treatments. The AP tanks showed slightly higher densities of rotifers on average during Experiment 2, which may have contributed to the higher abundance of bacteria in the water. The characteristic initial proliferation of bacteria, including an increase in the fraction of opportunists, was more pronounced in the AP tanks than in the LA or CL tanks. The microbial abundance remained low in the CL tanks due to the reduced concentration of organic matter, i.e., a lower CC. Tanks with live *I. galbana* maintained low numbers of

bacteria despite the higher levels of organic matter, which may be due to the presence of antibacterial compounds. Microalgae may promote or inhibit bacterial growth by production of active metabolites or by providing a selective regime for bacteria by the release of DOM (Duff *et al*., 1966, Bruce *et al.*, 1967, Kogure *et al*., 1979, Kellam and Walker, 1989). Björnsdóttir (2010) reported that addition of clay resulted in lower numbers of cultivable bacteria in water and halibut larvae compared to when marine microalgae was added. In agreement with this, a higher number of CFU was found in LA water than CL water after three days in tanks fed rotifers but without larvae (Experiment 2), and LA showed higer total bacteria cell counts than CL in Experiment 3.

Low numbers of bacteria and a low CC in itself are not automatically beneficial. The composition of the microbial community is in many cases more decisive for the performance of larvae than the absolute abundance of bacteria (Vadstein *et al*., 1993, 2004, Munro *et al*., 1995, Verner-Jeffreys *et al.*, 2004). A high share of fast-growing opportunists and *Vibrio* spp. is, however, considered to be negative for the performance of young marine larvae (Vadstein *et al*., 1993, 2004, Munro *et al*., 1994, Skjermo *et al*., 1997, Nicolas *et al*., 1989, Hansen and Olafsen, 1999, Skjermo and Vadstein, 1999, Verner-Jeffreys *et al.*, 2003, Samuelsen *et al*., 2006, Sandlund and Bergh, 2008, Reid *et al*., 2009). An increase in the fraction of cultivable bacteria is common when substrate levels increase and may be understood as a transition from a less active and oligotrophic state to a more rapidly growing state in eutrophic systems (Ruby and Morin, 1979). At a similar CC, a low CB may also indicate relatively higher amounts of specialists (as opposed to opportunists) present (Skjermo *et al*., 1997). The fraction of TCBS counts and CB were higher in AP tanks than for the other treatments. Compared to LA tanks, there was a higher fraction of opportunists and TCBS counts in CL and AP tanks, suggesting a suboptimal development in the microbial composition.

The LA tanks were considered to offer the most beneficial microbial environment for the larvae because the addition of live *I. galbana* resulted in low fractions of opportunistic bacteria and TCBS counts in both the water and rotifers. Addition of *Isochrysis* sp. has been found to inhibit proliferation of opportunists in the rearing water of turbot (*Scophthalmus maximus*) (Salvesen *et al*., 1999). Moreover, extracts from microalgae have been shown to inhibit growth of several opportunistic pathogens of fish and shellfish, including many *Vibrio* spp. (Austin and Day, 1990, Austin *et al*., 1992, Naviner *et al*., 1999). *Vibrio* spp. are rarely associated with microalgae cultures (Lewis *et al*., 1988, Salvesen *et al*., 2000). Incubation

with *Tetraselmis* sp. has been used to reduce the number of bacteria and *Vibrio* spp. and increased the relative diversity of bacteria associated with *Artemia franciscana* (Olsen *et al.*, 2000).

Opportunistic bacteria are frequently found in rotifer cultures (Skjermo and Vadstein, 1993). The relative abundances of opportunists and TCBS counts in rotifers in Experiment 2 closely reflected the microbial composition of the water of the respective treatments. The concentration of CFU in the rotifers was similar in all treatments, in agreement with results reported by Nicolas *et al.* (1989) and Skjermo and Vadstein (1993). The composition of the bacterial community in both water and live feed has implications for the colonization of gut flora of larvae (Munro *et al.*, 1993, 1994, Reid *et al.*, 2009).

4.3 Effects on the performance of fish larvae

As discussed by Vadstein *et al.* (1993, 2004), the effect of microbial mitigation is expected to be greatest when fish larvae are stressed or if their performance is compromised by other factors. During the first days following hatching, the larvae are sensitive and in a critical stage for microbial colonization of gut and skin.

Microbial factors may give plausible explanations for a great part of the differences in the performance of fish larvae in our first feeding experiment. The growth of larvae seemed to be related to the general microbial community composition, whereas survival appeared to be more influenced by specific microbial incidents. LA tanks were generally considered to maintain the best microbial environment, with low numbers of bacteria and low proportions of TCBS counts and opportunists in water and rotifers. Tanks with clay also showed low numbers of bacteria, but a higher fraction of opportunists, which may be harmful to larvae. Application of algae paste resulted in what was interpreted to be an inferior microbial composition with higher bacterial abundance and a higher proportion of TCBS counts, opportunists, and cultivable bacteria. It is important that CL and LA larvae initiated exponential growth earlier than AP larvae, because early initiation of exponential growth is considered to reflect high quality of marine larvae (Reitan *et al.*, 1993, Skjermo *et al.*, 1997).

It was striking that the filamentous bacteria got such a foothold in the LA tanks compared to the other treatments. The filaments did not seem to influence larval growth significantly, but

may have caused the high mortality observed in the LA tanks. The larval mortality at 13 dph in one of the replicate CL tanks could be related to the conspicuously higher concentration of TCBS counts (5×10^3 mL^{-1}) observed in that particular tank at 3 dph. In comparison, the survival of halibut larvae reared in water with clay was similar to that of larvae in water with marine microalgae in a first feeding experiment by Björnsdóttir (2010).

Clay is probably present in some of the natural habitats of cod larvae, like in Trondhjemsfjorden, but as far as we know, little is known of physiological effects of adding clay to the rearing water. Pelagic marine fish larvae depend in part on cutaneous respiration which may be a reason why any negative effect on gill epithelium does not seem critical at the earliest stage. High concentrations of silicate (>40 g L^{-1}) or kaolin clay (2-3 g L^{-1}) did not cause direct gill damage, but induced stress responses in juvenile coho salmon (*Oncorhynchus kisutch*) and steelhead (*Salmo gairdneri*) (Redding *et al*., 1987, Lake and Hinch, 1999). In the presented experiment, cod larvae apparently ate and grew well in tanks with clay, but the long-term effects of this treatment are not known.

The growth of the cod larvae was as fast in the tanks with clay as in the other treatments, which indicates that clay addition did not negatively affect the nutritional value of the rotifers. In a preliminary trial with clay addition to the rearing water of cod larvae (unpublished data), rotifers were observed to disappear faster than in tanks with clear water. The rotifer density was only slightly reduced by the presence of clay in the absence of fish (Experiment 2). This suggests that the observed higher rate of rotifer disappearance in CL tanks with larvae was mainly a result of increased ingestion rates rather than a result of a negative interaction with clay particles. It has been hypothesized that organic matter and bacteria, adsorbed to clay particles, may contribute to the nourishment of oyster spat (Matson *et al*., 2006). Ingested clay particles may have effects on the bacteria community of the digestive tract of the fish. Björnsdóttir (2010) reported differences in the bacterial community composition in halibut larvae reared in water with clay compared to marine microalgae. She exclusively observed two species of bacteria belonging to *Marinomonas* spp. and *Shewanella* spp. in halibut rearing water with marine microalgae, and one species belonging to *Polaribacter/Flavobacteriaceae* in water conditioned with inorganic clay.

Interaction with bacteria is only one of several factors affecting survival, growth, and quality of larvae. The effect may be masked by other factors influencing the performance. Treatments

that affect the microbial environment may also have direct positive or negative effects on larvae. As an example, even though the turbidity was in the same range in our experiment, it was, on average, higher in the tanks with clay, and the different treatments may have resulted in differences in light conditions, which could have affected foraging and performance of larvae.

4.4 Implications

Although the general microbial load was reduced, a lowering of bacterial substrate levels by substituting algae with clay appeared to still leave an opening for opportunists and possible pathogens to colonize the rearing environment at an early stage. The microbial community of the intake water was probably not granted the time to mature and stabilize before bacterial substrate, rotifers, and larvae were introduced to the tanks. Selective enhancement of the bacterial environment through microbial maturation and controlled recolonization of the intake water is a complementary method for microbial control improving the performance of fish larvae during the first days of rearing (Vadstein *et al.*, 1993, Skjermo *et al.,* 1997, Salvesen *et al*., 1999). The method allows the biomass of the microbial community to adjust to the CC post treatment by promoting succession and stabilisation during some retention time in a reservoir with a large surface area occupied by slow-growing bacteria. The amount of free niches available for opportunistic growth is then reduced. However, when microbial maturation is targeted at the relatively low microbial CC of intake water, the abrupt transition to higher substrate levels in the rearing tanks still represents a potential opening for opportunistic proliferation. To close the gap in microbial CC between intake water and rearing tanks, Salvesen *et al*. (1999) encouraged the development of systems with the capacity to produce matured water at a CC matching that of the rearing tanks. This should theoretically reduce the amount of open niches and the chance of proliferation of opportunistic microbes in the rearing tanks. Turning it around, lowering the CC of the rearing water to match that of matured intake water could have a similar effect. Microbial maturation of intake water combined with the addition of clay to the rearing tanks could be an efficient and easy way to gain microbial control in a flow through system for marine larvae.

The type and species of clay and algae used in this study were chosen on the basis of availability and common usage in commercial hatcheries for marine fish. Different species of algae have different properties in both live cultures and as concentrates, and it can not be

ruled out that species other than the ones we used may have different effects. For example, cultures of *I. galbana* were associated with a high share of slow-growing bacteria, whereas live *N. oculata* cultures were found to contain slightly more haemolytic and opportunistic bacteria in a study by Salvesen *et al.* (2000). The results might have been more similar in the algal treatments if the live culture and paste was of the same species. In contrast to our experiment, no differences were found in the performance of larval cobia reared in algae paste compared to live microalgae (Schwarz *et al.*, 2008). In a similar way, different types of clay have different properties with respect to level of purity, particle size, cation exchange capacity, adsorption capacity and swelling ability. The type and source of the clay may affect the removal of bacterial substrate. Nevertheless, our study illustrated some general properties of the different substances used to create turbidity in early marine larval rearing and may be used as a basis for further development of the method.

Substituting algae paste with clay can limit high abundance of bacteria by reducing the load of organic matter, and hence reduce the microbial CC, at a stage when larval sensitivity is high and the effect of external water treatment is limited. Clay addition is cheaper and simpler than application of both algae paste and live algae, and it seems to be a good alternative in terms of cost and performance in the early stages of the production of marine fish larvae.

5 Conclusions

1. A rearing regime for cod larvae with addition of clay resulted in reduced levels of bacterial substrate in the rearing water compared to rearing regimes with live algae or algae paste. This was mainly a result of the reduced input of organic matter in the tanks with clay.

2. Clay aggregated dissolved organic matter in the water and transported it to the bottom of the rearing tanks. About 5 % of the small and dissolved organic matter in the tanks could be removed daily with the debris.

3. Fish tanks with algae paste showed higher abundance of bacteria and a higher share of cultivable bacteria and TCBS counts than tanks with clay or live algae. Tanks with live algae had low proportions of opportunistic bacteria and TCBS counts in both water and rotifers.

4. Cod larvae, in tanks with clay or live algae, initiated exponential growth earlier, and had higher growth rates than larvae in tanks with algae paste.

5. Addition of clay is cost efficient compared to the application of live algae or algae paste.

6. Clay addition, in combination with microbial maturation of intake water, may contribute to a more stable microbial community in the larval rearing tanks.

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Paper 4

Biotic and abiotic particles protect marine heterotrophic bacteria during UV and ozone disinfection

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ABSTRACT: Disinfection of water is required for a range of applications, including ballast water treatment and land-based fish farming. Bacteria attached to or embedded in particles can be protected from the disinfectant by various mechanisms. We investigated inactivation of marine heterotrophic bacteria in the presence of biotic and abiotic particles. In one set of experiments with the planktonic rotifer *Brachionus* 'Nevada', water was exposed to increasing UV and ozone dose, and we examined inactivation of free-living and particle-associated heterotrophic bacteria. An estimated 99.9% inactivation of free-living bacteria was obtained compared to only 91.4% inactivation (3 of 4 experiments) of particle-associated bacteria at the same ozone dose. For the UV experiments, a 6-fold increase in disinfection dose was required to obtain 99.9% inactivation of the particle-associated compared to the free-living bacteria. In a second set of experiments we investigated the protective effect of biotic (rotifers) and abiotic (ceramic clay) particles as a function of particle concentration. Increased particle concentration resulted in reduced disinfection efficiency of free-living bacteria with both UV and ozone. Rotifers protected slightly better against UV disinfection than ceramic clay particles, while such a relationship was not evident for the ozone disinfection. The results suggest a complex bacterial inactivation mechanism in the presence of particles, and will have implications for the treatment strategy used for ballast water and land-based fish farming.

KEY WORDS: Disinfection · Bacteria · Inactivation · Ozone · UV · Seawater · Biotic particles · Abiotic particles

INTRODUCTION

Invasive species and pathogens can be introduced to natural ecosystems through sources like ballast water from ships and effluents from aquaculture. One way to reduce the transfer into new locations is to disinfect the water. UV irradiation and ozonation are 2 disinfection methods used in such situations; however, water used as ballast or in aquaculture can contain a wide range of type and concentration of particles which may interfere with the disinfection process.

Ozone (O_3) is a powerful oxidising agent and has been used as a treatment for the inactivation of organisms such as bacteria, in freshwater and seawater. By introducing ozone to seawater, a series of redox reactions take place and several reactive intermediates are formed. The main ozone demand in seawater is due to its reaction with bromide to form bromine compounds. The most important reaction in seawater during ozonation is the initial oxidation of bromide ions (Br^-) to hypobromite ions (OBr^-) which can then be reduced back to Br^- or further oxidised to form bromate ions (BrO_3^-) (Buchan et al. 2005). The hypobromite ion will hydrolyse into hypobromous acid (HOBr), which is a weak acid. The sum of HOBr and OBr^- is the biocidal bromine. In seawater with a typical pH of 8, hypobromous acid will predominate and be the most important disinfectant with a half-life of hours to days dependent on light conditions and water quality characteristics (Liltved et al. 2006). Dissolved ozone levels can be esti-

mated indirectly by measuring the oxidation–reduction potential (ORP) or by the colorimetric N,N-diethyl-p-phenylenediamine (DPD) method. ORP may be used to control ozone addition to seawater but is not necessarily equivalent to the disinfective capacity of the water (Tango & Gagnon 2003). Buchan et al. (2005) recommend the DPD 'total chlorine test' be used and express the results as total residual oxidant (TRO), in units of mg Cl_2 l^{-1}. The disinfection dose is commonly calculated from the average TRO concentration during the contact time and gives the $C \cdot t$ value (i.e. the product of concentration and contact time).

The disinfective ability of UV irradiation is due to harmful changes in DNA molecules and mainly due to radiation in the UV-C spectral region. Light with wavelength around 254 nm will damage DNA and RNA by photo-induced dimerisation of adjacent pyrimidine in the nucleic acid strand. Due to covalently bound pyrimidine residues, replication of the nucleic acid is hindered or completely blocked. This effect can be temporal or lethal depending on the repair mechanisms and the degree of UV resistance. Many bacteria have developed mechanisms, some of which are dependent on light, with which to repair moderate damage. Photo reactivation is attributed to the ability of light in the wavelength range 330 to 480 nm to activate DNA photolyase. In the dark, excision repair has been shown to be the important mechanism. These recovery processes may be limited, but should not be neglected when assessing the efficiency of disinfection. Particular concern should be aimed at the processes that take place after discharge to the recipient water (Liltved & Landfald 1996).

Bacteria adhered to or embedded in particles may obtain protection against chemical and non-chemical disinfection agents, with reduced disinfection efficiency as the result. This protection mechanism can act differently depending on the disinfection method used. The effect of disinfection by radiation such as UV can be reduced by particles shielding, absorbing, scattering and blocking of UV light from the bacteria. The effect of chemical disinfection agents can be reduced due to disinfectant degradation at the particle surface and rate-limited transport of disinfectants into the particle (Perrins et al. 2006a). Bacteria colonising the particle surface or inside, similar to the biofilm system, are known to be more resistant than free-living bacteria to chemical disinfection at lower concentrations of disinfectant (Wu et al. 2005). Many bacteria are known to be particle-associated and this could be a strategy to survive unfavourable conditions. The protective mechanisms will depend on particle properties such as size, type and concentration. To our knowledge the protective mechanisms of particles in seawater are poorly studied. We tested the following hypotheses: (1) parti-

cles will protect embedded or attached bacteria from disinfectants; (2) disinfection efficiency of free-living bacteria is negatively correlated to particle concentration; (3) biotic particles give better protection of heterotrophic bacteria than abiotic particles; and (4) the properties of the disinfection methods cause UV disinfection to be more affected by particle concentration than ozone disinfection.

The present study was designed to give further insight into inactivation of heterotrophic bacteria in seawater and the protective effect of particles by ozone and UV. This was done by studying dose-inactivation relationships of free-living and biotic (rotifers) particle-associated bacteria, and by comparing protection by biotic and abiotic particles (ceramic clay) as a function of particle concentration.

MATERIALS AND METHODS

Particles and seawater. Two types of disinfection experiments were performed: dose experiments with biotic particles, and a particle concentration experiment with biotic and abiotic particles. The planktonic rotifer *Brachionus* 'Nevada' (size ~250 μm) was used as the biotic particle and was collected from a batch culture (250 l conical vessels in seawater of 20‰ salinity at 20°C) with densities between 44 and 394 ind. ml^{-1}. *Brachionus* 'Nevada' is 1 of 4 distinct phylogenetic lineages within group A of the *B. plicatilis* species complex as suggested by Gómez et al. (2002). This rotifer can grow in a wide range of salinities and temperatures, and rapid shifts result in only temporary changes in swimming ability (Øie & Olsen 1993). Mixing of cultures during treatment secured an even distribution of rotifers. Ceramic clay (Vingerling k 148, WBB Fuchs) was used as the abiotic particle. In the disinfection dose experiments, the rotifer culture was diluted (final salinity, 28 to 30‰) in autoclaved seawater to obtain appropriate densities to examine dose-dependent inactivation of particle-associated and free-living bacteria. In the particle concentration experiment, washed rotifers (biotic) and ceramic clay particles (abiotic) were added to the rotifer culture water (45 μm filtrate) in increasing amounts and diluted in autoclaved seawater before inactivation of free-living bacteria were compared. The seawater (34‰ salinity) used in these experiments was collected from Trondhjem Biological Station's high-quality influent water (intake at 70 m depth), aged (>2 mo in the dark at ~15°C) and autoclaved before use.

Disinfection procedures. Ozonated water was produced by injection of ozone into the experimental mixture through an air stone to a stable ORP of 400 mV controlled by automatic set point with 3 s delay in a 1 l

beaker with continuous stirring. ORP was measured and controlled using Redoxpotential-Steuergerät (Erwin Sander). Ozone was produced by a Sander Ozonisator Ozoniser (2000 mg h^{-1}; Erwin Sander) supplied with air from an air pump (air pump N022AN.18, KNF Neuberger). Samples were taken by pumping the required volume through a 0.4 cm (inner diameter) tube using a peristaltic pump into a sterile 50 ml Falcon tube at given time intervals. TRO was measured immediately after each sample withdrawal.

The water subjected to UV treatment was placed in the reaction chamber (640 ml) of a UV water disinfection system (Aquapro 1GPM, 220V/14W, Aquapro Industrial) with continuous stirring. Samples were taken by emptying the reaction chamber into a sterile glass bottle. The average intensity (14.57 mW cm^{-2}) at the quartz glass wall was measured by a UVX-25 Radiometer (Ultraviolet Products). The UV dose at the mid-point inside the reaction chamber was calculated based on Beer Lambert's law:

$$I_x = \frac{\varphi}{2\pi r l} 10^{-Ax}$$

I_x is the radiation energy at distance x from the quartz tube (mW cm^{-2}), φ is the measured UV output at 254 nm multiplied by the surface of the quartz glass (mW), A is the absorbance by the irradiated water, x is the distance from the quartz tube to the mid-point in the chamber, l is the effective length of the UV-arch tube and r is the distance from the lamp centre to the mid-point of the reaction chamber (cm). Absorbance at 254 nm was measured with a spectrophotometer and the average absorbance in each experiment was used in the calculations. The UV dose was defined as the product of average intensity in the reaction chamber and the exposure time ($I_x \cdot t$).

Experimental design. In the dose experiments, variable doses for both disinfection methods were achieved by varying the exposure time, and the dose is expressed as C·t (mg s l^{-1}) for ozone and mJ cm^{-2} for UV. The particle concentration effect was studied at a UV dose of 586 mJ cm^{-2} and a C·t value of 30 mg s l^{-1} (DPD 0.25 mg Cl$_2$ l^{-1}). Biotic particles (0 to 171 mg DW l^{-1} rotifers) were collected by filtering the rotifer culture on a 45 µm mesh and rinsing the rotifers 5 times in sterile seawater (34‰ salinity). Numbers (rotifers and eggs) were converted to dry weight (DW) biomass, assuming 578 ng DW ind.$^{-1}$ and 228 ng DW egg^{-1} (Øie et al. 1997). Increasing abiotic particle concentrations (0 to 133 mg DW l^{-1} ceramic clay) were achieved by adding dried ceramic clay (110°C for 24 h). The particles were added to culture water, incubated for 15 min at room temperature, diluted 2-fold in autoclaved seawater to specified densities and exposed to treatment for 2 min. In the dose experiments, samples

were taken at time zero (2 samples), and after 0.17, 1, 2, 3, 4, 7, 15 and 30 min of exposure. In Expt Oz1, the samples were taken after 0.17, 1.5, 2.5, 2.8, 3.6, 4.1, 10 and 40 min of exposure.

The release of bacteria from rotifers to the water during the UV and ozone treatments was estimated by adding thoroughly rinsed rotifers to autoclaved seawater prior to disinfection.

Analytical procedures. To determine the number of particle-associated and free-living bacteria, a 50 ml sample of the treated water or of the culture was taken and fractionated in two by filtering through a 45 µm mesh. The filtrate represented the free-living bacteria and the concentrated rotifer solution represented the bacteria associated with the rotifers. Culture water of rotifers has been shown to contain little bacterial activity in the size fraction >1 µm (Vadstein et al. 1993), which supports our rationale for classifying the fraction <45 µm as free-living bacteria.

The concentrated rotifers were washed 2 times with a minimum of 50 ml sterile seawater (34‰ salinity) to remove free-living bacteria. A subsample (2 or 3 ml) of the washed and concentrated rotifers was homogenised. The filtrate (both experiments) and the homogenised rotifers (only the dose experiments) were serially diluted in sterile seawater (34‰ salinity) and spread out on M65-agar plates (0.5 g peptone, tryptone, yeast extract, 15 g agar, 800 ml aged seawater and 200 ml tap water) to determine the number of colony-forming units (CFU) as an estimate of total numbers of culturable bacteria. CFU was determined after incubation of the agar plates in the dark at 15°C for 14 d by mean of a colony counter (AcoLyte), and expressed as CFU ml^{-1} or CFU rotifer^{-1} for free-living and particle-associated bacteria, respectively. The agar plates were prepared in triplicate, and the average CFU results are presented in this paper. The majority of counted agar plates used for quantification had 20 to 200 CFU ml^{-1}, giving an SD of 4 to 13% per triplicate. Close to the detection limit, lower numbers of CFU were observed, giving a higher SD as a consequence. A subsample of the washed and concentrated rotifers was fixed in acid Lugol's solution and rotifers were manually quantified with a dissecting microscope.

Chemical and physical variables were determined by withdrawal of an additional sample (30 ml). Measurements of temperature, salinity (salinity refractometer ATC-S/Mill-E, ATAGO), pH (standard meter, PHM210, Radiometer Analytical), absorbance (Ultrospec 2000 UV/Visible spectrophotometer, Pharmacia Biotech) and TRO (ozone treatments) were performed for each sample, including the time zero samples (t_0). In addition, temperature and ORP (mV) were monitored directly in the ozone reaction beaker. TRO were measured by the colorimetric DPD method immedi-

ately after sample withdrawal using a HACH DR 890 colorimeter. TRO is expressed as mg Cl_2 l^{-1}, and the $C \cdot t$ value was calculated from the average TRO concentration during the exposure time. The zero samples (t_0) were used as blanks for the DPD measurements and autoclaved filtered seawater was used as a blank for the absorbance measurements.

RESULTS

Time-zero conditions

The dose-response relationship for particle-associated and free-living bacteria was studied in 3 separate experiments for the UV treatment and in 4 separate experiments for the ozone treatment. Expts Oz4 and UV4 were performed the same day and with the same culture. This was also done in Expts Oz5 and UV5 and in the particle concentration experiment (ozone and UV). Time-zero conditions and experimental set-ups are presented in Table 1. The initial (t_0) CFU of the free-living bacteria ranged from 2.7×10^6 to 2.0×10^7 ml^{-1} in the experiments, corresponding to a $7.4 \times$ variation. The particle-associated CFU ranged from 5.0×10^3 to 1.12×10^5 CFU rotifer^{-1} at t_0, a 22× variation. If Expt Oz2 is excluded, the range will be reduced to a factor of 6.8 and 3.9, respectively. The variation in free-living bacteria was partially caused by the variable dilution of the rotifer culture.

Inactivation of bacteria

In the dose-response experiments, particle-associated bacteria showed a significantly higher survival rate than free-living bacteria in all ozone experiments (Fig. 1). Thus bacteria experienced a high degree of protection when associated with rotifers. The inactivation of free-living bacteria also levelled off at high $C \cdot t$
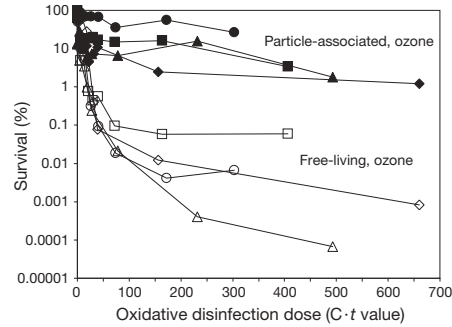

Fig. 1. Survival versus dose response for free-living (open symbols) and particle-associated (solid symbols) bacteria in ozonated rotifer cultures. Expts Oz1 (◇, ◆), Oz2 (○, ●), Oz4 (△, ▲) and Oz5 (□, ■)

values, but this can be explained by CFU approaching the detection limit. In general the parallel ozone experiments followed a similar trend; the most deviating $C \cdot t$ observation in each experiment deviated from the median by a factor <2. The only exception was Expts Oz1 and Oz5 at 90% inactivation of free-living and particle-associated bacteria, respectively (excluded from the kinetics calculations). The average kinetics are presented in Table 2. A 90% reduction in survival was observed at an average $C \cdot t$ value of 4.1 and 11.5 mg s l^{-1} for the free-living and the particle-associated bacteria, respectively. In 3 out of 4 experiments, 99.99% inactivation of free-living bacteria was obtained at an average $C \cdot t$ value of 138.2 mg s l^{-1}, whereas the maximum inactivation was 98% for the particle-associated bacteria (Fig. 1).

In the UV irradiation experiments, particle-associated bacteria also had higher survival rates than free-living bacteria (Fig. 2). However, the protection due to particle association was much lower than in the ozone experiments, and inactivation levelled off at higher doses for both free-living and particle-associated bacteria, probably due to CFU approaching the detection limit. A 99.9% reduction in survival of free-living and particle-associated bacteria was achieved for all the parallel UV experiments at similar doses (the most deviating dose deviated from the median by a factor of 1.3); on average, a dose of 350 mJ cm^{-2} and 2.12 J cm^{-2} was needed, respectively. A 99.99% inactivation of free-living bacteria was achieved for an average dose of 1.61 J cm^{-2}, and 2 out of 3 particle-associated experiments achieved the same

Table 1. Time zero average counts (CFU) of free-living, particle-associated bacteria and rotifers in the water subjected to ozone or UV treatment

Expt	Culture dilution	Free-living (CFU ml^{-1})	Rotifer-associated (CFU rotifer^{-1})	Rotifers ml^{-1}
Oz1	2-fold	6.21×10^6	2.86×10^4	35
Oz2	5-fold	1.06×10^6	2.04×10^3	55
UV3	5-fold	5.98×10^6	1.69×10^4	79
Oz4/UV4[a]	2-fold	2.00×10^7	9.98×10^4	28
Oz5/UV5[a]	2-fold	7.78×10^6	1.12×10^5	22
Particle conc. Oz/UV[a]	2-fold	2.96×10^6		[b]

[a]Experiments were performed the same day and with the same culture
[b]To the particle concentration experiment, 0 to 171 mg DW l^{-1} rotifers and 0 to 133 mg DW l^{-1} ceramic clay was added

Table 2. Percentage inactivation by ozonation (4 experiments) and UV irradiation (3 experiments) of free-living and particle-associated bacteria. Data are mean ± SD. na = not applicable (this level of inactivation was not achieved)

Inactivation (%)	Sample	Ozone dose (mg s l^{-1})	UV dose (J cm^{-2})
90	Free-living	4.13 ± 2.37[a]	0.05 ± 0.02
	Particle-associated	11.50 ± 3.25[b]	0.11 ± 0.12
99	Free-living	21.85 ± 4.29	0.18 ± 0.04
	Particle-associated	na	0.44 ± 0.38
99.9	Free-living	48.88 ± 15.85	0.35 ± 0.03
	Particle-associated	na	2.12 ± 0.44

[a]Based on 3 out of 4 experiments
[b]Based on 2 out of 4 experiments

reduction with approximately 7.55 J cm^{-2} (Fig. 2). A similar dose increase was required to obtain 90% inactivation of the particle-associated compared to the free-living bacteria for both the UV and ozone experiments. The same was observed for the stepwise (90 to 99 to 99.9%) inactivation of the free-living bacteria with both treatments.

The degree of protection of particle-associated bacteria can be expressed as relative percent protection (RPP = 100% × [1 – (inactivation of the particle-associated bacteria/inactivation of the free-living bacteria)]). The average RPP in each experiment at elevated doses varied between 5.4 and 46.1% for the ozone experiments and between 0.05 and 0.2% for the UV experiments (Fig. 3). If Expt Oz2 is excluded, the ozone experiments are more similar (5.4 to 12.7%). On average a 16-fold increase in dose gave a 5.5-fold decrease in RPP for the ozone experiments (Expt Oz2 excluded), and for the UV experiments corresponding

Fig. 3. Relative protection of bacteria by particles in ozone- and UV-treated rotifer culture given as relative percent protection (RPP). Symbols represent treatment time, not doses: 4 (◇), 7 (□), 15 (△) and 30 min (○). RPP = 100% × [1 – (inactivation of the particle-associated bacteria/inactivation of the free-living bacteria)]

numbers are 7.5-fold increase and 27-fold decrease. In general each dose step gave a corresponding decrease of RPP in the UV experiments (except Expt UV3), but to a variable degree. This relationship was not evident in the ozone experiments, but the highest dose gave the lowest RPP in each experiment.

The experiment evaluating the effect of concentration of biotic and abiotic particles on survival of free-living bacteria after UV and ozone treatments is presented in Fig. 4. An effect of particle concentration was observed for both treatments, but with no clear differences between particle types. For the ozone treatment, survival increased from 1.5% with no particles, to 20%
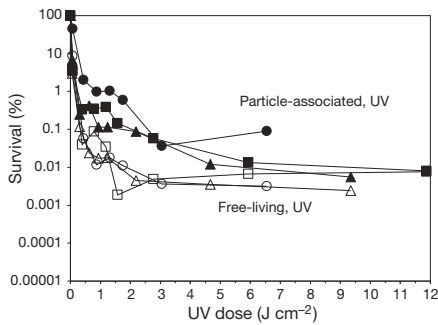

Fig. 2. Survival versus dose response for free-living (open symbols) and particle-associated (solid symbols) bacteria in UV-irradiated rotifer cultures. Expts UV3 (○, ●), UV4 (△, ▲) and UV5 (□, ■)

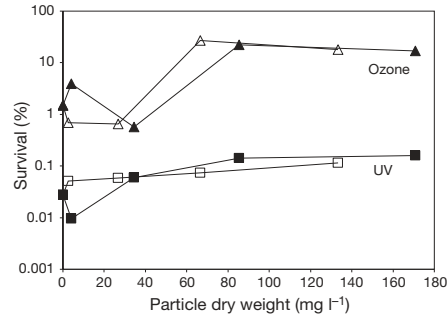Fig. 4. Effect of biotic (rotifers, solid symbols) and abiotic (ceramic clay, open symbols) particle concentration on survival of bacteria after UV (□, ■) or ozone (▲, △) treatment. The water was treated for 2 min, giving an average UV dose of 586 mJ cm^{-2} and an average C·t value of 30 mg s l^{-1}

for particle concentrations above 65 and 85 mg DW l^{-1} for the abiotic and biotic particles, respectively. For the UV treatment, survival increased from 0.03% with no particles, to 0.1% for particle concentrations above 110 and 65 mg DW l^{-1} for abiotic and biotic particles, respectively. This corresponds to a ~13- and ~3-fold increase in survival, for the ozone and UV treatment, respectively. For both treatments there was a deviation from the pattern at 4 mg DW l^{-1} of biotic particles, but we found no apparent reason for this deviation. The release of bacteria from rotifers to the water during the UV and ozone treatments was low and had an insignificant impact on survival (data not shown). Data presented in Fig. 4 are therefore not corrected for the release of live bacteria from rotifers.

DISCUSSION

The results obtained demonstrate reduced UV and ozone disinfection efficiency for both particle-associated and free-living bacteria in seawater containing particles. However, the protection was considerably higher for the particle-associated bacteria. Inactivation of free-living bacteria by the 2 disinfection methods responded differently to increased particle concentrations, with no or minor differences between biotic and abiotic particles. Previous studies on disinfection of bacteria in seawater use 2 different approaches, either testing organisms in pure culture (Sugita et al. 1992, Liltved et al. 1995) or heterotrophic communities of bacteria (Liltved & Cripps 1999, Leonard et al. 2000, Sharrer et al. 2005, Herwig et al. 2006, Perrins et al. 2006a,b, Masters et al. 2008). Reported UV and ozone doses needed to reach a specific inactivation level vary tremendously between these 2 approaches. There may be several reasons for this difference and some will be discussed here. Direct comparison of disinfection doses using these 2 approaches should be avoided, and care should also be taken when comparing results using the same approach because the experimental set-up and bacterial detection methods vary. Inactivation dose data for the free-living bacteria obtained in the present study are in the same range as those published for communities of heterotrophic bacteria. Such studies report larger variation in inactivation doses than the pure culture studies. Reported disinfection doses using the community approach and our results are significantly higher than the doses recommended in aquaculture (Wedemeyer 1996, Liltved 2002). Earlier studies (Liltved & Cripps 1999, Wu et al. 2005) of particle effects on UV-irradiated water also demonstrate protection of bacteria, but little is known about the effects of increased disinfection dose (UV or ozone), particle concentration, and particle type on the inactivation of heterotrophic bacteria in seawater. Based on our data, it is possible to elaborate on the differences in inactivation of free-living and particle (biotic and abiotic)-associated bacteria by UV irradiation and ozonation of seawater.

The difference observed in CFU for both free-living and particle-associated bacteria at t_0, especially in Expt Oz2, may be related to the variable status of the rotifer cultures. It has been shown that densities of rotifer-associated and free-living bacteria vary considerably depending on total grazing pressure, defecation and feeding regime (Skjermo & Vadstein 1993, Vadstein et al. 1993, O. Vadstein unpubl.).

The dose-response results demonstrated a significantly higher survival rate of bacteria associated with rotifers. For both methods, a 2-stage inactivation was observed, which is a deviation from first-order kinetics (Figs. 1 & 2). Inactivation of particle-associated bacteria in the ozone experiments was less affected by increased dose than the UV experiments. The low initial TRO concentration (ORP) or insufficient contact time can also contribute to the slow inactivation (Sugita et al. 1992, Perrins et al. 2006a,b), possibly making the C·t value insufficient to describe dose. Liltved et al. (1995, 2006) showed inactivation curves of fish pathogenic bacteria and viruses in seawater exposed to UV irradiation and ozonation close to and deviating from first-order kinetics, respectively. The inactivation curves levelled off at higher survival for the particle-associated than for the free-living bacteria. This can be explained by either protection of the bacteria in the digestive tract of the rotifer or the presence of populations with higher disinfection tolerance associated with rotifers. Comparative studies of the bacterial flora in water and associated with rotifers showed strong correlation (Skjermo & Vadstein 1993), making the presence of bacteria with higher tolerance associated to rotifers less likely. Protection by particles expressed as RPP indicates different protection at elevated doses for the 2 disinfection methods (Fig. 3). The dose increase had a greater effect on RPP for the UV-treated than for the ozone-treated bacteria, indicating a TRO threshold level for the ozone dose. This may be different at higher ORP (initial TRO concentrations). The higher survival rates of UV- and ozone-exposed bacteria associated with rotifers, even at very high disinfection doses, indicates mechanisms to escape disinfection.

Protection of bacteria in the shadow of other particles seems likely for UV irradiation (Liltved & Cripps 1999), but should be absent with ozonation, where consumption or a rate-limited transport of disinfectants into the aggregates may be the protection mechanism. We hypothesised that biotic particles would give better protection than abiotic particles for both treatments,

with larger differences between the biotic and abiotic particles for the UV treatment than for the ozone treatment. Effects like shielding, absorption and scattering in the UV experiment was believed to reduce the UV treatment efficiency more than the consumption and rate-limited transport of disinfectants in the ozone treatment. Our results from the particle concentration experiment demonstrated increased survival with increasing particle concentration for both particle types and disinfection methods, but with a more pronounced protection for the ozone treatment and little difference between the biotic and abiotic particles (Fig. 4). The increased survival with increasing particle concentration of ozone-treated bacteria (13-fold at maximum particle concentration) is believed to be caused mainly by the inactivation of disinfectants by the particles before bacteria. Thus, increased survival can also partly be attributed to the low TRO concentration (0.25 mg s l^{-1}) and insufficient contact time. Both types of particles exhibited little protection (3-fold at maximum particle concentration) during UV irradiation of bacteria, indicating weaker protective properties against UV irradiation. The inactivation of disinfectants by particles in the ozone experiment seems to protect the bacteria better than the shading by particles in the UV experiment. The efficiency of UV and ozone treatment may also be affected by the contact time between particles and bacteria prior to treatment (Wu et al. 2005), but is believed to be of minor importance in our experimental conditions and for the free-living bacteria. Increased particle concentration was not clearly detected with UV absorbance measurements, but increased protection was observed. Particles in water may have different absorbance and scattering properties, influencing measurements of UV absorbance and determination of the fluence rate. Some particles may increase the fluence rate and others like clay and organic matter may reduce the fluence rate (Mamane et al. 2006). This may influence conventional measurements of suspended particles; therefore measurements of scattering should be incorporated in order to more accurately reflect absorbance in UV disinfection systems.

Lasting inactivation of bacteria may be impossible to achieve, and one has to consider the time span for which the disinfection effect should last. Further work on recolonisation kinetics of disinfected water by heterotrophic bacteria should be performed to gain further knowledge on the long-term effects of disinfection. For treatment of e.g. ballast water and effluents from land-based fish farming, particle properties and concentrations should be evaluated to set a sufficient disinfection dose and/or in combination with particle removal. This will improve the overall bacterial inactivation efficiency and reduce the risk of introducing alien species and possible spreading of pathogenic bacteria.

CONCLUSIONS

This study examined the protective mechanisms provided by biotic and abiotic particles during UV and ozone inactivation of heterotrophic bacteria in seawater. Our results support the hypothesis that bacteria embedded in or attached to particles are protected, and only partially support the hypothesis that disinfection efficiency of free-living bacteria is negatively correlated to particle concentration. There was little difference in protection between the particle types. However, the biotic particles protected bacteria slightly better during UV treatment, and increasing particle concentration reduced the ozone disinfection efficiency more than the UV disinfection efficiency. Thus the hypothesis that biotic particles give higher protection than abiotic particles is supported by the UV experiment, but not the ozone experiment. Our results do not support the hypothesis that disinfection by UV is more affected by particle concentration than ozone disinfection. The ozone experiments show a large difference in inactivation of free-living versus particle-associated bacteria and demonstrate particle protection of free-living bacteria. The UV experiments show less difference in inactivation of free-living versus particle-associated bacteria and little particle protection of free-living bacteria. The overall results confirm differing protection kinetics and mechanisms for heterotrophic bacteria for the 2 disinfection methods used.

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1999 Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis

1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen, I	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems

2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar

2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	ph.d Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	ph.d Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	ph.d Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	ph.d Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	ph.d Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	ph.d Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation

2006 Kari Mette Murvoll	ph.d Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds
		Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006 Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006 Nils Egil Tokle	ph.d Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006 Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006 Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006 Johanna Järnegren	ph.d Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006 Bjørn Henrik Hansen	ph.d Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006 Vidar Grøtan	ph.d Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006 Jafari R Kideghesho	ph.d Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006 Anna Maria Billing	ph.d Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006 Henrik Pärn	ph.d Biology	Female ornaments and reproductive biology in the bluethroat
2006 Anders J. Fjellheim	ph.d Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006 P. Andreas Svensson	ph.d Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007 Sindre A. Pedersen	ph.d Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007 Kasper Hancke	ph.d Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007 Tomas Holmern	ph.d Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007 Kari Jørgensen	ph.d Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007 Stig Ulland	ph.d Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007 Snorre Henriksen	ph.d Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007 Roelof Frans May	ph.d Biology	Spatial Ecology of Wolverines in Scandinavia
2007 Vedasto Gabriel Ndibalema	ph.d Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania

2007	Julius William Nyahongo	ph.d Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	ph.d Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	ph.d Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	ph.d Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	ph.d Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	ph.d Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	ph.d Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	ph.d Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, (*Taeniopygia guttata*)
2008	Sølvi Wehn	ph.d Biology	Biodiversity dynamics in semi-natural mountain landscapes. - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	ph.d Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"
2008	Katarina Mariann Jørgensen	Dr.Scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	ph.d Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	ph.d Bilogy	*Arabidopsis thaliana* Responses to Aphid Infestation
2008	Jussi Evertsen	ph.d Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	ph.d Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	ph.d Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	ph.d Biology	Cost of rapid growth in salmonid fishes
2008	Line Johansen	ph.d Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	ph.d Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease

2009	Pål Kvello	ph.d Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	ph.d Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	ph.d Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches
2009	Zsolt Volent	ph.d Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	ph.d Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	ph.d Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	ph.d Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments -Focus on formulated diets
2010	Eli Kvingedal	ph.d Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	ph.d Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	ph.d Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. Tha Case of Serengeti Ecosystem, Tanzania
2010	Anton Tinchov Antonov	ph.d Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	ph.d Biology	Population Ecology of *Eriophorum latifolium*, a Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	ph.d Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	ph.d Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	ph.d Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters
2010	Hans-Richard Brattbakk	ph.d Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	ph.d Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	ph.d Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	ph.d Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS:– The science of space experiment integration and adaptation to simulated microgravity
2011	Aline Magdalena Lee	ph.d Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics
2011	Christopher Gravningen Sørmo	ph.d Biology	Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in *Arabidopsis thaliana*

2011 Grethe Robertsen	ph.d Biology	Relative performance of salmonid phenotypes across environments and competitive intensities
2011 Line-Kristin Larsen	ph.d Biology	Life-history trait dynamics in experimental populations of guppy (*Poecilia reticulata*): the role of breeding regime and captive environment
2011 Maxim A. K. Teichert	ph.d Biology	Regulation in Atlantic salmon (*Salmo salar*): The interaction between habitat and density
2011 Torunn Beate Hancke	ph.d Biology	Use of Pulse Amplitude Modulated (PAM) Fluorescence and Bio-optics for Assessing Microalgal Photosynthesis and Physiology
2011 Sajeda Begum	ph.d Biology	Brood Parasitism in Asian Cuckoos: Different Aspects of Interactions between Cuckoos and their Hosts in Bangladesh