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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 favor 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. The survival of larvae in the tanks added clay was variable. 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. It is not possible to conclude whether this was related to the use of clay or an incidental development of a harmful microbial community in this tank. The effects of clay addition on larval performance should be studied further. 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;

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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 colonization of the fish gut (Bergh et al., 1994).

Visual feeders like cod larvae may benefit from turbidity increasing prey contrast in the water (Utne-Palm, 1999). Other sources of turbidity, like commercially available microalgae pastes and ceramic clay, are used in many hatcheries because they are labor- 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, 1992; 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, and rotifers are generally not used as live feed for halibut, 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.

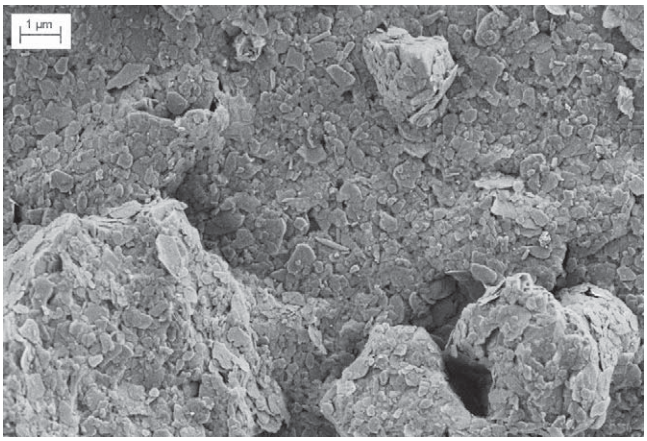

Fig. 1. Field emission microscopy picture of the ceramic clay (photo by Tor A. Nilsen).

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, CA) 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 illite mineral. The dose of clay added was chosen on the basis of the resulting turbidity in tanks (~10 cm Secchi depth in Experiments 2 and 3, which was the level reported by the halibut production industry). 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. The ceramic clay used consisted of leaf like particles less than 1 μm in size (Fig. 1), thus giving the clay a high surface area to weight ratio.

Sea water was pumped from 60 m depth in Trondhjemsfjorden (pH 8.0, 34 ppt salinity, 2 mg L^{-1} total organic carbon, TOC) 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 Experiments 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 analyzed 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 analyzed for bacteria after 3 and 6 days.

2.1.3. Experiment 3

Experiment 3 was a first feeding experiment, and Fig. 2 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 min 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 with the morning and afternoon feeding. No clay was added with the midday feeding as the water in the CL tanks still remained turbid from the previous addition at this point of time. 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).

2.2. Analytical procedures

2.2.1. Organic matter and turbidity

DOC and POC samples were immediately vacuum filtered through ignited (480 °C, 2 h) 0.7 μm, 25 mm diameter GF/F glass microfiber filters (Whatman International Ltd., England). Filtrate and filters were stored at −20 °C. Filtrate was analyzed 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). The optical density, OD, at 750 nm, was measured in a Shimadzu double-beam spectrophotometer (UV-150-02) and used as a measure of turbidity.

2.2.2. Colony forming units

Rotifers were concentrated on a 50 μm 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 ± 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 characterized 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 4 °C. Two mL of sample was diluted with 3 mL milli-Q water and vacuum filtered onto black polycarbonate filters (0.22 μm 25 mm diameter, Poretics Corp., USA) 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 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 (60 °C, 48 h), and analyzed in a CHN Elemental Analyser 1106 (Carlo Erba Instruments, Italy) at 1020 °C. 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 Eqs. (1) and (2):

$$\mathrm{SGR}(\mathrm{d}^{-1}) = \frac{\ln \mathrm{DW}_t - \ln \mathrm{DW}_0}{t - t_0} \quad (1)$$

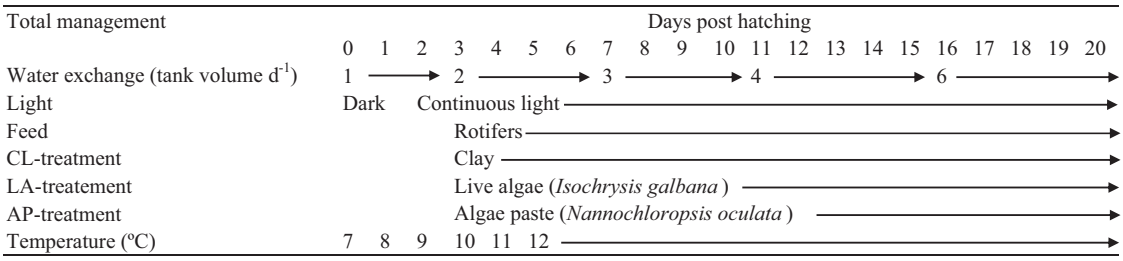

Fig. 2. Rearing regime, Experiment 3.

$$\%SGR\,(d^{-1}) = (eSGR^{-1}) \times 100\% \quad (2)$$

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

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 (Fig. 3). 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 Fig. 3, 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.

3.2. Experiment 2

In Experiment 2, the optical density (at 750 nm) of the culture water was in the same range for all treatments when measured before the addition of particles in the morning, but higher in tanks with clay ($p < 0.001$, Fig. 4). 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, Fig. 5). However, on average over the experiment, the CL tanks tended to have lower densities of rotifers than the AP tanks ($p = 0.035$, manual counts, Fig. 5a). AP tanks showed a higher density of rotifers than the two other treatments on average for the period with automatic rotifer counts ($p < 0.001$, Fig. 5b). It should be noted that the observed trend may unintentionally originate from other sources than particle addition, for example the feeding procedure.

In Experiment 2, which did not involve fish larvae, AP tanks showed a higher density of colony forming bacteria in the water

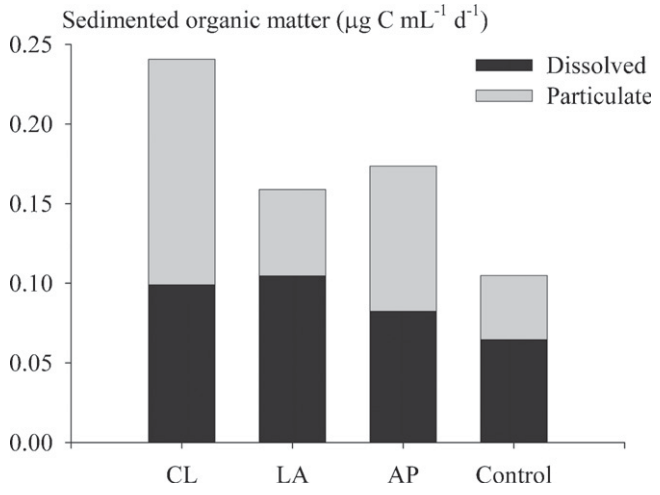

Fig. 3. The amount of dissolved (black) and particulate (gray) organic matter sedimented to the 1 cm of bottom water per mL of rearing water per day in tanks with yeast extract (control) and clay, live algae or algae paste (Experiment 1).

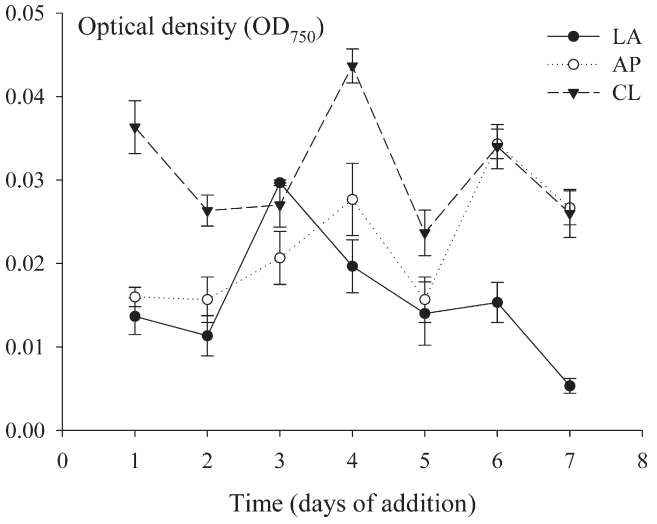

Fig. 4. Absorbance (OD_{750}) in tanks receiving live algae (●), algae paste (○), and clay (▼) for one week (Experiment 2).

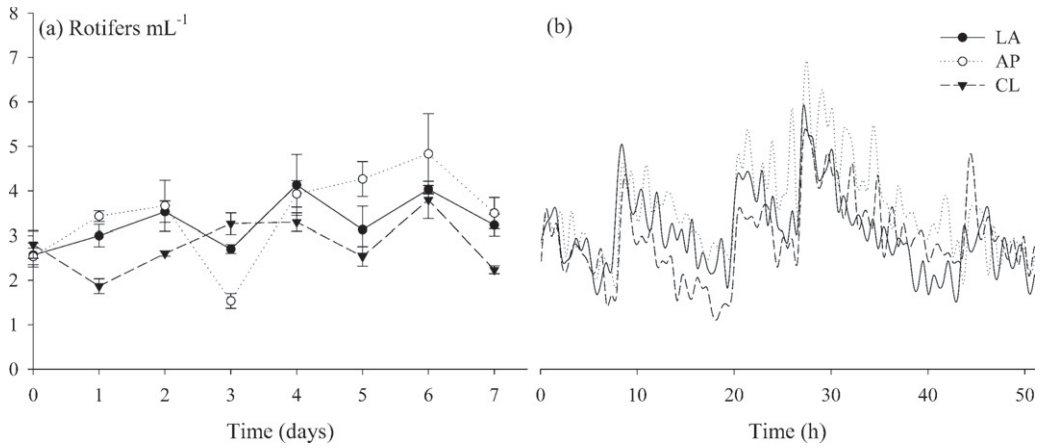

Fig. 5. Rotifer density (a) counted manually for 6 days and (b) counted automatically for the 50 first hours of the experiment, in the absence of fish in tanks receiving live algae (solid line, ●), algae paste (dotted line, ○), or clay (dashed line, ▼) in Experiment 2.

than the CL tanks ($p = 0.024$, Fig. 6a). CFU per rotifer was similar in all treatments (Fig. 6a). 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 (Fig. 6b and c). AP tanks showed a higher fraction of TCBS counts in the water than the other two treatments ($p = 0.017$, Fig. 6c), and both the AP and CL tanks showed relatively high fraction of TCBS counts and opportunists in the rotifers after three days.

3.3. Experiment 3

In the first feeding experiment, the concentration of DOC and POC increased as a result of the addition of live algae and algae paste (Fig. 7). 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 ($p < 0.001$).

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, Fig. 8a 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, Fig. 8c). 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. The AP tanks and one of the CL tanks also showed a higher fraction of TCBS counts of total CFU than the tanks of the other treatments through Experiment 3 ($p < 0.019$, Fig. 8d).

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 (Fig. 9a). 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.

Cod larvae in the CL and LA tanks initiated exponential growth earlier than AP larvae (Fig. 9b). 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 1.

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 (Utne-Palm, 1999). Generally, rotifers and fish larvae did not seem to be adversely affected by the clay in our experiments. However, one cannot rule out the possibility that the crash of one of the CL fish tanks was connected to the addition of clay. On the contrary, the performance of larvae in tanks with clay was good. Table 2 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 1
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

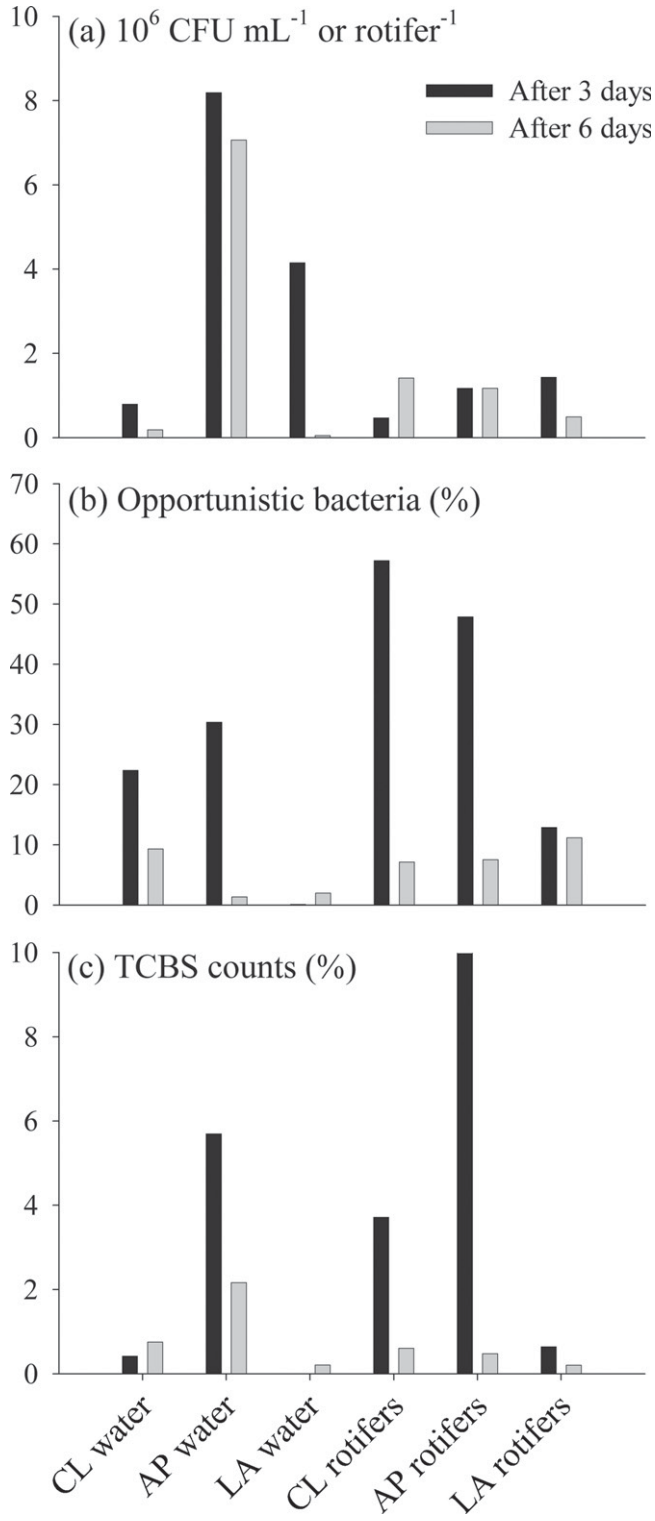

Fig. 6. (a) Colony forming bacteria, (b) the fraction of opportunistic bacteria and (c) the fraction of TCBS counts of total CFU in water and rotifers of tanks receiving clay, live microalgae, or algae paste in the absence of fish after 3 (black) and 6 (gray) days of particle addition (Experiment 2).

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 (Fig. 7).

Table 2
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	Variable

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 h 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

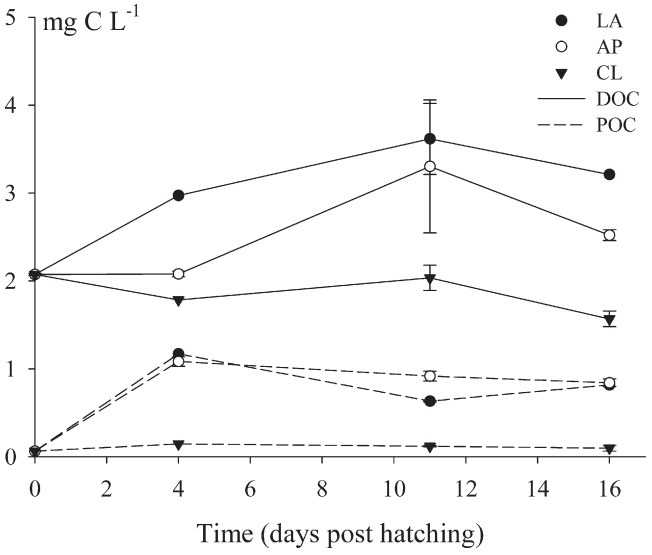

Fig. 7. Dissolved organic carbon, DOC (solid line), and particulate organic carbon, POC (dashed line), in fish tanks receiving live algae (●), algae paste (○), and clay (▼) in Experiment 3.

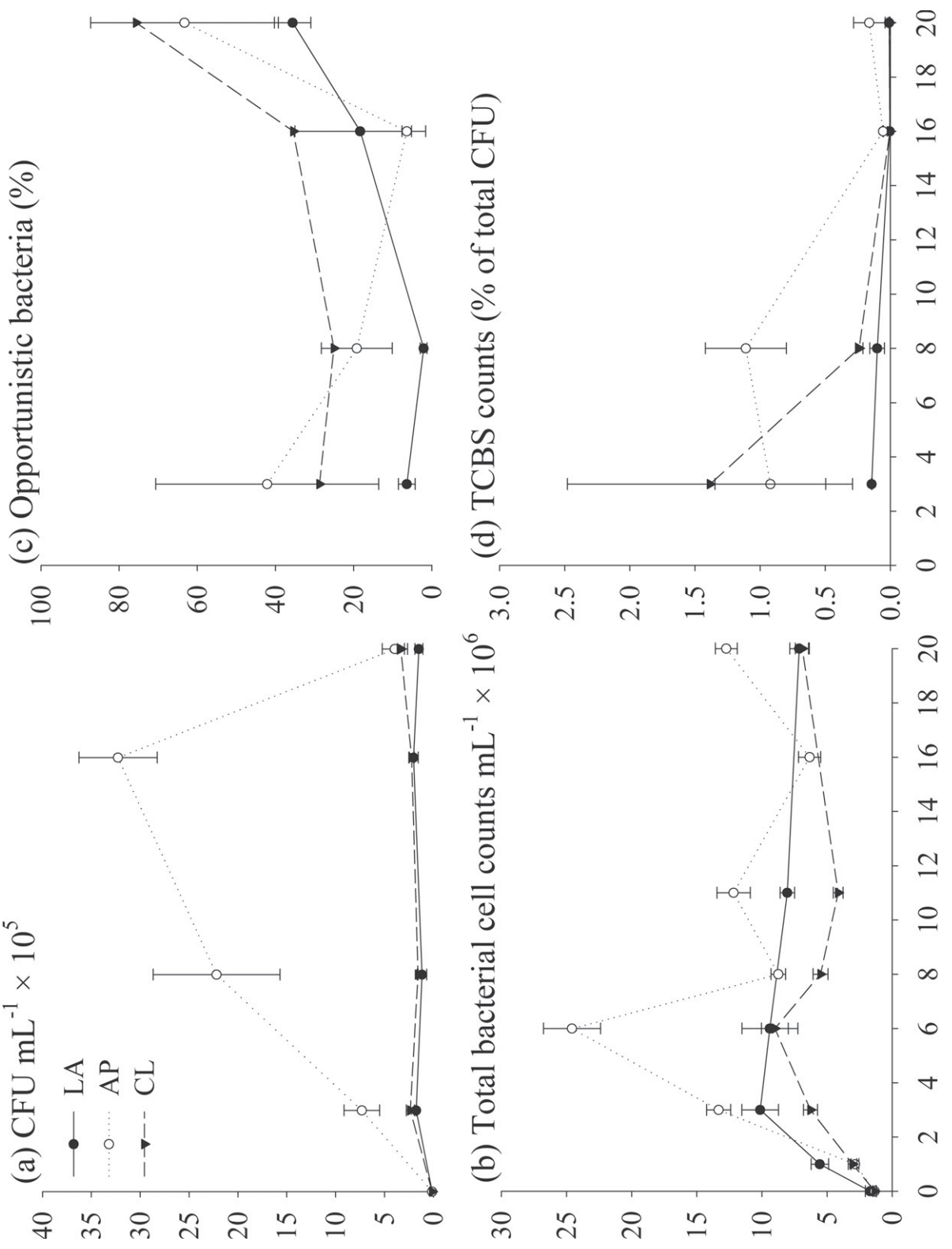

Fig. 8. (a) Colony forming units, CFU, (b) total bacterial cell numbers, (c) the fraction of opportunistic bacteria and (d) the fraction of TCBS counts of total CFU in the fish tanks receiving live algae (●), algae paste (○), and clay (▾) in Experiment 3.

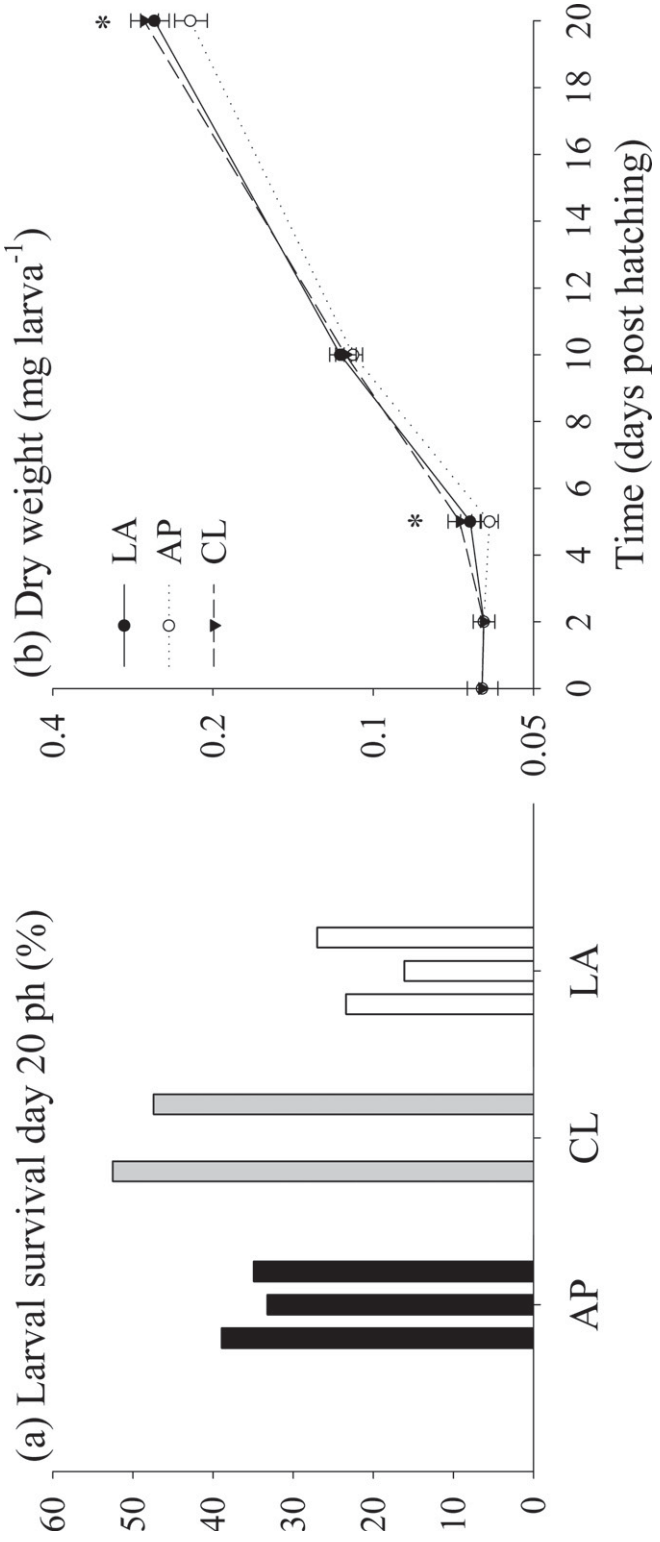

Fig. 9. (a) Survival and (b) growth of cod larvae reared in water receiving live algae (●), algae paste (○), and clay (▾) in Experiment 3. Significant difference is marked with an asterisk in (b).

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 (<50 μm) 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 destabilize 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 favors 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. Also, a higher fraction of the POC was comprised of intact algal cells that were probably less exposed to bacterial growth than algal fragments in the algae paste. 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 higher 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 fraction of cultivable bacteria (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. LA and CL, which were the treatments that showed low total numbers of bacteria in the tank water, initiated exponential growth earlier than AP larvae. This is important, because early initiation of exponential growth is considered to reflect high quality of marine larvae (Reitan et al., 1993; Skjermo et al., 1997).

In this experiment, the CL tank that crashed showed elevated numbers of TCBS counts some days before the total mortality was observed. Crashes of rearing tanks are not uncommon, and are often attributed to negative fish–microbe relationships. The feed and the measured physicochemical conditions were similar for all tanks, which may indicate that it was a negative development in the microbial community in the specific tank that crashed that affected the larvae. There is certainly a possibility that the mortality of this CL tank is connected with the addition of clay to the water, but the results still show that for certain conditions (the two other replicate tanks) clay addition may give very good survival and growth. It is possible that the addition of clay increases the chances for crashes of rearing tanks, but it is not possible to draw any conclusions about that from this experiment. On the other hand, stabilization (microbial maturation) of the water could potentially have reduced the chance of this kind of crashes, but was not applied in this experiment. Little is known of the effects of clay on larval performance and further research is needed to clarify questions like this. 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).

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.

Clay is probably present in some of the natural habitats of cod larvae, 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). 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. Closing the gap in microbial CC between intake water and rearing tanks should theoretically reduce the amount of open niches and the chance of proliferation of opportunistic microbes in the rearing tanks (Salvesen et al., 1999; Attramadal et al., 2011). One way to accomplish this may be to lower the CC of the rearing water with the use of clay to match that of matured intake. In this way, 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 cannot 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 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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