The use of ozone in a high density recirculation system for rotifers

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Received 4 May 2000; accepted 12 January 2001

Abstract

The use of ozone in the effluent treatment of a closed recirculation system for rotifers resulted in a significant improvement of rotifer production and water quality. Compared to a control treatment, the rotifer culture exposed to ozone did not only support a higher rotifer biomass (16000 vs. 8000 rotifers ml⁻¹), it also allowed a prolongation of the culture period for 4 days. Compared to a control treatment, the ammonium levels were reduced by 67%, nitrite levels by 85% and nitrate levels by 67%. The supplementation of ozone did not affect pH and dissolved oxygen levels. Besides the positive effect of ozone to the nitrification process, a better removal of suspended solids was noticed as well. This resulted in a decreased turbidity and a reduction of the number of particles in the culture water. The use of ozone also reduced the number of bacteria in the culture water.

In general terms, it can be stated that supplementation of ozone in a closed recirculation system for rotifers considerably improves water quality, ensures stable and longer rotifer culture periods and controls bacterial proliferation. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Brachionus plicatilis; Recirculation; Ozone; Rotifers
1. Introduction

The rotifer *Brachionus plicatilis* is an excellent first food for fish and crustacean larvae, but there are still some problems related to its culture and use. The most stringent problems for automation of the production cycle reside in the unpredictability of the mass production and the variability in the quality of the product (Walz et al., 1997). The unstable production and low quality of rotifers produced in commercial hatcheries can mainly be explained by the static culture procedures (batch cultures) in which water quality is degrading rapidly. This gradual deterioration of the water undoubtedly affects rotifer quality and is also at the origin of the variability in larval performance (Oie et al., 1997). Recently, new culture methods in rotifer production have been developed enabling high density populations in continuous culture (Abu-Rezq et al., 1997; Fu et al., 1997; Yoshimura et al., 1997; Suantika et al., 2000). Suantika et al. (2000), Yoshimura et al. (1994) and Snell et al. (1987) demonstrated that water quality was a first prerequisite for healthy rotifer cultures but no attention was made to the hygienic quality of rotifers. However, it is assumed that rotifers, the first food administered to fish larvae, are the major carriers of bacteria (Munro et al., 1993, 1994) causing poor survival and growth of fish larvae (Gatesoupe, 1989). For this reason, the reduction of bacteria and/or a method of controlling bacterial populations in rotifer cultures needs to be considered in rotifer production units.

In our study, we evaluated the use of ozone as a disinfectant in a recirculation rotifer culture system as a tool to improve water quality and reduce the bloom of opportunistic bacteria. Ozone is a colourless gas that has been used in several aquaculture systems to disinfect or sterilize water supplies or discharge systems (Owsley, 1991; Cryer, 1992), it is also applied to control bacterial proliferation in closed recirculation systems. It was used by Bullock et al. (1997) in a recirculating rainbow trout (*Oncorhynchus mykiss*) culture system to reduce the numbers of heterotrophic bacteria and to prevent bacterial gill disease (BGD) in newly stocked fingerlings. Also, Summerfelt et al. (1997) investigated the advantages of ozone and its impact on filtration and water quality in a fish culture system.

2. Materials and methods

2.1. Rotifer strain

All experiments were performed with *B. plicatilis* (L-strain with lorica length: 180 ± 15 μm). Before the start of the experiment, the rotifer strain was kept in culture at the Laboratory of Aquaculture and Artemia Reference Center, following the culture procedure described in Sorgeloos and Lavens (1996).

2.2. Experimental set up

The rotifers were inoculated at a density of 500 individuals ml⁻¹ in 100 l PVC cylindro-conical tanks in three replicates per treatment. The culture water consisted of diluted seawater (25 ppt salinity), maintained at a constant temperature (25 ± 1°C).

The rotifer rearing system was connected to a closed water recirculation system (Fig. 1). The effluent water was drained from the rotifer culture tank, purified from suspended solids and soluble protein in a protein skimmer before being filtered over a submerged biofilter. After the biological filtration, the treated water was reinjected into the rotifer culture tanks at a daily water renewal rate of 500% (Suantika et al., 2000).

Ozone (O₄) was produced by two ozonisers (Sander Ozonisator 100 and 200, Sander Aquariatechnik, Germany) connected in series on the suction line of the venturi of the protein skimmer. In order to improve the efficiency of the ozoniser, the incoming air was dried through 700 g silica gel that was renewed every 24 h. Residual O₃ was removed from the effluent water by an active carbon filter (10 l capacity) placed before the biofilter. The control experiments were run using identical equipment except ozone injection into the protein skimmer. The biofilters were seeded with nitrifying bacteria (10⁹ CFU ml⁻¹; ABIL, Aqua, Avecom, Belgium) 6 days before the inoculation of rotifers in the culture tank. During these 6 days, the performance of the biofilter was monitored by daily ammonium, nitrite and nitrate measurements to make sure that no difference in biological activity was induced. The second run experiment was conducted under the same condition except for the conditioning of the biofilter. In this experiment, 20 l (~ 30 kg) CaCO₃, and 215 mg l⁻¹ NH₄Cl were added to the biofilter to improve the buffer capacity of the biofilter (Fig. 2).

Fig. 1. Schematic overview of the recirculation system.
storage tanks, the food was administered automatically by means of a peristaltic pump to the individual rotifer culture tanks at 1-h interval (feeding 33 ml h⁻¹ or 800 ml food suspension day⁻¹). The rotifers were fed following a standard feeding regime (Suantika et al., 2000):

\[ CSH = 0.035D^{0.415}V, \]

where: CSH = the weight of experimental diet (g), D = rotifer density (individuals ml⁻¹), V = culture water volume (l).

2.5. Physico-chemical parameters

The pH, NH₄⁺, NO₃⁻, NO₂⁻, ORP (oxygen reduction potential) and the dissolved oxygen (DO) of the water were measured as first daily activities during the experiment. NH₄⁺, NO₃⁻, and NO₂⁻ were performed on the culture water of the rotifer tanks filtered through a 30-μm filter. The NH₄⁺, NO₃⁻ and NO₂⁻ concentration were determined using test kits (Aquamerck®; Viscolor Eco®, Germany). The ORP was measured at the outflow of the protein skimmer and before the biofilter as well as in the rotifer culture using a redox meter (Smarest™ Series-Model ORPScan, Singapore). The biological oxygen demand (BOD) and the chemical oxygen demand (COD) were measured in the rotifer culture tank, in the protein skimmer, and after the biofilter on day 5 of the culture period by using Winkler titration (BOD) and potassium dichromate (COD) method. The absorbance was measured by using a spectrophotometer at 600-nm wavelength.

2.6. Bacterial sampling

Water samples (10 ml) were taken from the culture and the protein skimmer after 5 days of rotifer culture. For plating, serial dilutions were prepared in sterile saline solution (1.5%) from the homogenised water samples. Four solutions of 100 μl were plated in duplicate on MA (marine agar) (Difco, USA) and TCBS (thiosulphate citrate bile sulphate agar) (Difco) and incubated at 25°C. Bacterial counts were performed at 24 and 48 h incubation.

2.7. Statistical analysis

All experiments were performed in triplicates and repeated three times. All data were statistically treated using two-way ANOVA. Significant differences among means (\( P < 0.05 \)) were tested by Duncan’s multiple range test.

3. Results

3.1. Conditioning of the biofilter

The performance of the biofilter during the 6-day conditioning period is presented in Fig. 2. In the first run, the biofilter was conditioned by gradually increasing the daily

2.3. Sampling and counting

Three samples of 500 μl were taken from the rotifer cultures using an automatic micropipette. The rotifers in each sample were killed by adding three drops of lugol and counted. Empty and transparent loricae belonging to dead rotifers were not counted.

Suspended organic matter (e.g. excess food, flocules, etc.) from the effluent water trapped in the foam of the protein skimmer was removed daily and subjected to microscopic observation. The organic waste particles were counted using a haematocytometer (Fulch-Rosenthal) following the method described in Sorgeloos and Lavens, 1996.

2.4. Rotifer diet

The rotifer diet consisted of Culture Selco High, CSH (INVE, Belgium) suspended in 800-ml water and mixed vigorously with a kitchen blender. The suspension containing exactly the daily food ratio was kept in cold storage (4°C) for 24 h. From the cold
addition of NH₄⁺ (110 mg l⁻¹ for the total period of 6 days). No significant difference in biological activity was observed between the biofilter for the control treatment and the one for the ozone treatment. In the second run, the daily amount of NH₄⁺ was increased during the conditioning period of 6 days (325 mg l⁻¹). Also, in this second run, the biological activity of the biofilter for the control treatment and the one used for the ozone treatment was not significantly different.

3.2. Rotifer growth performance

The average growth rate of the rotifer populations reared in the recirculation system for the control treatment and the treatment with ozone supplementation is presented in Fig. 3. In the two replicated experiments, a significant increase in the rotifer density (P < 0.05) was obtained when ozone was supplemented. In the control treatment, the culture collapsed on day 11 approximately 1 day after a maximal rotifer density of 7300 rotifers ml⁻¹ was reached. The addition of ozone in the recirculation system allowed a prolongation of the culture period of 4 days. In the ozone treatment, a maximal rotifer density of 15 000 rotifers ml⁻¹ was obtained on day 14. No significant differences in rotifer density were obtained in the repeated experiment of the control treatment. For the ozone treatment, however, the average maximal rotifer density was significantly different (15 200 rotifers ml⁻¹ for trial I and 20 500 rotifers ml⁻¹ for trial II) (Table 1). The highest rotifer production was obtained in the repeated experiment where the biofilter was better conditioned (better buffering) with CaCO₃ and higher NH₄⁺ supply.

3.3. Physico-chemical parameters

The physico-chemical parameters did not show significant differences among the repeated trials (except for nitrile) and have been grouped as average values in Fig. 4.

At the beginning of the culture period, the ozone and control treatment had a pH level of 8.0. From day 1 onwards, the pH level decreased slowly without any significant difference among the treatments and replicated experiment (Fig. 4A).

Lower and more stable ammonium levels were observed during the culture period in the systems exposed to ozone in which the NH₄⁺ level fluctuated from 0.3 to 0.6 mg l⁻¹ (Fig. 4B). In the control, the NH₄⁺ level was double as high as the ozone treatment and ranged from 0.7 to 1.2 mg l⁻¹.

Low nitrite levels (NO₂⁻) were obtained in the beginning of the culture period in both treatments (from day 0 to day 4). The NO₂⁻ level remained stable and low (NO₂⁻ < 2.5 mg l⁻¹) in the system exposed to O₃ during the complete culture period (Fig. 4C). On the other hand, the NO₂⁻ level increased drastically from day 5 onwards in the control treatment. Lower nitrite levels were obtained during the repeated experiment with better biofilter conditioning.

Low nitrate (NO₃⁻) levels were observed in the beginning of the culture period (from day 0 to day 5). From day 6 onwards, the NO₃⁻ level increased drastically in the control treatment. A slower and steady increase was measured in the system exposed to ozone compared to the control treatment during the first 10 days (Fig. 4D).

### Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Experiment I Control</th>
<th>Ozone</th>
<th>Experiment II Control</th>
<th>Ozone</th>
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<td>500 ± 0⁰a</td>
<td>500 ± 0⁰a</td>
<td>500 ± 0⁰a</td>
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<td>1632 ± 97⁰b</td>
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<td>4509 ± 83⁰a</td>
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<td>7</td>
<td>5275 ± 338⁰a</td>
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<td>5784 ± 1015⁰a</td>
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<td>6017 ± 663⁰a</td>
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<td>11239 ± 777⁰</td>
<td>15086 ± 2173⁰</td>
<td>20344 ± 1937⁰</td>
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<tr>
<td>12</td>
<td>13276 ± 850⁰</td>
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<td>18119 ± 2246⁰</td>
<td>20344 ± 1937⁰</td>
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<tr>
<td>13</td>
<td>14028 ± 1261⁰</td>
<td>14028 ± 1261⁰</td>
<td>20544 ± 1937⁰</td>
<td>20544 ± 1937⁰</td>
</tr>
</tbody>
</table>

Means within the same row and followed by the same letter are not significantly different (P > 0.05).
During the culture period, dissolved oxygen slightly decreased from 5.7 to 2.6 mg l\(^{-1}\) (during 13 days) and 5.8 to 3.7 mg l\(^{-1}\) (during 10 days) for the system exposed to ozone and without supplementation of ozone, respectively (Fig. 4E).

A lower water colouration and turbidity was observed in the ozone treatment and is reflected in the figures obtained from the analysis of the water samples in the spectrophotometer (Fig. 4F).

In the system exposed to ozone, the protein skimmer reduced the chemical oxygen demand (COD) from 231 mg l\(^{-1}\) in the culture water to 91 mg l\(^{-1}\) and reduced the biological oxygen demand (BOD) from 25 to 6 mg l\(^{-1}\). In the control treatment, the protein skimmer reduced the COD from 216 mg l\(^{-1}\) in the culture water to 136 mg l\(^{-1}\) and reduced the BOD from 15 to 10 mg l\(^{-1}\).

Fig. 5. Quantitative and qualitative evaluation of the skimming capacity of the protein skimmer exposed to a control and ozone treatment. (A) Effluent water collected from the protein skimmer. (B) Organic waste particles in the effluent water.
In the ozone treatment, ORP measurements ranged from 310 to 360 mV in the protein skimmer and 165–225 mV in the culture water (Fig. 5).

3.4. Quantitative determination of skimming capacity

The skimming capacity and the composition of the collected suspended solids from the protein skimmer in the recirculation system for the control treatment and the treatment with ozone supplementation is presented in Fig. 5. In the control treatment, 730 ml day\(^{-1}\) was collected on average from the protein skimmer. A three times larger amount of effluent water was collected from the control skimmer when ozone was supplemented (Fig. 5A). During the 10-day culture period, no difference in food losses were noticed between the control and the ozone treatment (1.1 \(\times\) 10\(^{11}\) and 3.2 \(\times\) 10\(^{11}\) particles, respectively). The total organic waste (4.1 \(\times\) 10\(^{11}\) particles collected from the control treatment and 5.7 \(\times\) 10\(^{12}\) particles collected from the ozone treatment), however, was significantly different (Fig. 5B).

3.5. Bacterial counts

The bacterial counts were performed 48 h after incubation of the plates. The use of ozone in the recirculation system reduced the amount of bacteria in the culture water (Table 2). In trial I, the amount of bacteria in the culture water for the system exposed to ozone was 2.7 \(\times\) 10\(^{4}\) and 10\(^{3}\) CFU ml\(^{-1}\) on marine agar and on TCBS, respectively. The bacterial counts measured in the protein skimmer were significantly reduced to less than 30 CFU ml\(^{-1}\) on marine agar and on TCBS. For the control treatment, no reduction on bacterial counts was noticed. The culture water contained 9.0 \(\times\) 10\(^{4}\) and 1.8 \(\times\) 10\(^{3}\) CFU ml\(^{-1}\) on marine agar and on TCBS, respectively, and 10\(^{2}\) and 5.4 \(\times\) 10\(^{2}\) CFU ml\(^{-1}\) in the protein skimmer.

In the second trial, the bacterial counts on marine agar were reduced from 2.7 \(\times\) 10\(^{7}\) to 8.0 \(\times\) 10\(^{3}\) CFU ml\(^{-1}\) in the protein skimmer and from 2.0 \(\times\) 10\(^{4}\) to 3.6 \(\times\) 10\(^{3}\) CFU ml\(^{-1}\) on TCBS. For the control treatment, the culture water contained 3.0 \(\times\) 10\(^{7}\) and 1.7 \(\times\) 10\(^{5}\) CFU ml\(^{-1}\) on marine agar and on TCBS, respectively, and 1.6 \(\times\) 10\(^{5}\) CFU ml\(^{-1}\) (marine agar) and 3.2 \(\times\) 10\(^{5}\) CFU ml\(^{-1}\) (TCBS) in the protein skimmer.

4. Discussion

Highly significant differences in the growth of the rotifer cultures were obtained by the use of ozone in the recirculation system. The application of ozone resulted in 1.3 times denser rotifer cultures in a 10-day rearing period. It also allowed a prolongation of the culture period for 4 days with an average standing populations of 1.6 billion rotifers per 100 l (trial I). Further fine tuning of the system in terms of biofilter conditioning resulted in a further increase in rotifer production of almost 40% (trial II).

Adding ozone to the recirculation system resulted in an overall improvement in water quality reducing nitrite levels by 85% and nitrate levels by 67%. The reduction of nitrite in the culture supplemented with ozone can be explained by the oxidative properties of ozone that rapidly oxidizes nitrite to nitrate (Rosenthal and Kruener, 1985). The addition of ozone in the recirculation system can thus be used on one hand to protect rotifers from toxicity/accumulation of nitrite; on the other hand, it can also be used to reduce the size of the biofilter. Especially in the recirculation system, where the conversion of nitrate to nitrite is the limiting factor, ozone can be applied efficiently. Although very few significant differences were obtained in the physico-chemical parameters, between the first and second culture trial, lower and especially more stable water parameters are at the origin of the better rotifer performance obtained in the second trial (Fig. 4). It is very likely that besides nitrite, the accumulation of other parameters, which are not significantly different on their own, may contribute to a higher toxicity when all factors were accumulated. It is difficult to estimate the effect of the higher concentrations of nitrate (and other components) on the life span of the rotifers, but the effect on reproduction rate and egg formation was clearly observed. Also, it should be observed that comparison between repeated experiments should be analyzed with care since slight differences in the quality of the rotifiers used as inoculum might result in different observations. As long as rotifer quality cannot be measured, we can not exclude that the better results obtained in the repeated trial are due to a better initial rotifer quality or even to differences in initial bacterial composition (Rombaut et al., 1999).

Bespoke the oxidative role of ozone in the nitrification process, a positive effect was also noticed on the coagulation–floculation of colloidal water substances (Farvardin and Collins, 1989). This results in an effluent water with better characteristics in terms of skimming capacity. In our experiment, this was measured in a decreased turbidity and a reduction of the number of particles in the culture water (Figs. 5 and 6). The result of the better skimming and oxidation obtained in the system supplemented with ozone can be illustrated in Fig. 6. Water in the rotifer rearing tank exposed to the ozone treatment has less abundant and smaller flocules and debris compared to the control treatment. This is explained by the repeated passages of the particles in the protein skimmer where they are partially oxidized by the ozone (reduction in size) and/or floculated (skimmed off) in the protein skimmer. Smaller and less abundant flocules and debris in the

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Bacterial counts obtained in the culture water and the protein skimmer for the control and ozone treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>Marine agar</strong></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>9.0 (\times) 10(^{4})</td>
</tr>
<tr>
<td>Protein skimmer</td>
<td>1.0 (\times) 10(^{3})</td>
</tr>
<tr>
<td><strong>TCBS</strong></td>
<td></td>
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<tr>
<td>Culture</td>
<td>1.8 (\times) 10(^{3})</td>
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<tr>
<td>Protein skimmer</td>
<td>5.4 (\times) 10(^{2})</td>
</tr>
</tbody>
</table>

Means within the same row and followed by the same letter are not significantly different (\(P > 0.05\)).
rearing system does not only result in better rearing conditions, it also considerably reduces the risk for clogging of the filters in the rearing tank.

The disinfecting properties of ozone also make it an ideal agent for the reduction of heterotrophic bacteria in the protein skimmer. In this experiment, the addition of ozone reduced the numbers of heterotrophic bacteria by $3\log_{10}$ units. A reduction in bacterial numbers can only have advantages in terms of the hygienic quality of the rotifers, however, attention should also be paid to the changes in bacterial communities imposed by the disinfection. In our experiment, no significant reduction of bacteria was observed in the culture water which can be explained by the retention time of the water ($\pm$ 5 h) which is longer than the bacteria doubling time. However, the continuous exposure of ozone could steer the microbial community in such a way that variation in the microbial community was far less important than in the batch system (Rombaut et al., 2000). Further increase of ozone supplementation can be applied in order to optimize the reduction of bacteria in the rotifer cultures; however, this aspect should be taken in consideration in determining an optimum pre-ozonation dose (OPD) in the system without any detrimental effect on the microflora community of the biofilter.

The addition of ozone in the recirculation system had no effect on the pH or dissolved oxygen concentration. The pH levels never reached below 7.0 during the experiment in both treatments. This stable pH is very important, not only for the rotifers but also for the optimal nitrification process in the biofilter.

It is surprising that although $O_3$ has several advantages, it is not more widely used. One of the main reasons that ozone is not widely used in aquaculture can be explained by its toxicity to aquatic organisms when overdosed (Bullock et al., 1997). Moreover, the lack of cheap and reliable instruments and chemical tests for online ozone detection in water probably explains the reluctance of several aquaculturist to apply it. Nowadays, however, indirect measurement of residual ozone by means of ORP is very reliable (Fig. 7). Based on this standard curve, the concentration of ozone obtained in the protein skimmer and in the culture were around 0.05 and 0.02 mg l$^{-1}$. These levels are safe for aquaria purposes and not toxic for the rotifers (Hemdal, 1992). A safe ORP in the culture water, for freshwater appears to be between 300 and 350 mV (Bullock et al., 1997). However, there is limited information on tolerance of rotifers to ozone and residual oxidants associated with the use of ozone in seawater. Davis and Arnold (1997) found that rotifers were sensitive to ozonated seawater, both as total residual oxidants consisting of ozone, chloramines, bromamines and residual oxidants excluding ozone. High levels of ozone ($\geq$ 1.63 mg l$^{-1}$) were found to inactivate rotifer eggs.

Summarizing, it can be stated that supplementation of $O_3$ in a closed recirculation system for rotifers considerably improves water quality, ensuring stable and longer rotifer culture periods. From a microbial point of view, reduced bacterial numbers were observed.

Acknowledgements

This study was supported by the Indonesian Government and managed through the center grant of the Department Biology-Institut Teknologi Bandung, under Contract No.
010/CG/II/URGE/1997, IBRD Loan No. 3754-IND. Part of this study was also supported by the FWO Project G. 006396 N.; CRAFT Project QSCR-2000-70186 and INVE, Belgium. The Laboratory of Microbial Ecology and Technology, Ghent University supplied the nitrifying bacteria and performed the BOD/COD analysis. The authors wish to thank T. Baelemans for his technical assistance during the experiment.

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