The influence of dietary oxolinic acid on fluidised bed biofilter performance in a recirculation system for rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The effect of medicated feed, containing 2.35 ± 0.01 mg oxolinic acid (OA) g⁻¹, upon the performance of fluidised bed (FB) biofilters was examined over a 29-day period. Rainbow trout were used as stock animals in duplicate recirculation systems. OA was detectable within the systems throughout the period of study, with bulk water OA concentrations reaching 1.24 mg l⁻¹ at their highest point. Sludge levels of OA were measured at 60 mg day⁻¹ at peak. A mass balance for OA was undertaken on day 21 of the study. Seventy-two percent of the antibiotic could be recovered from the trout, sludge and water, with fish being of minimal significance at trial termination. The treated feed did not effect biofilter performance, either during the feeding period 7 days duration or for 14 days post-feeding, with respect to nitrification rates. In separate studies, which employed a scale model of the recirculation system, maximum nitrification rates (MNR) were examined using media from each of the biofilters and four doses of OA (0.1–100 mg l⁻¹). OA did not effect MNR, but water turbidity and bacterial count increased. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Oxolinic acid; Fluidised bed; Nitrification; Sludge; Medicated feed; Mass balance

1. Introduction

Water recirculation systems provide many advantages, perhaps the three most important being: (1) conditions for cultured stock can be controlled for optimal growth;
(2) environmental effects can be reduced to fulfill the increased demand for cleaner production systems; and, (3) stock can be protected from external pollution events. However, increasing stocking densities must offset the costs involved in recirculation system setup. With increased stocking levels, comes a heightened risk of stock loss due to disease. Accordingly, recirculation systems generally incorporate components for water disinfection. Examples include UV-irradiation and ozonation (Liltved and Landfald, 1995). Nevertheless, bacterial infections remain a common problem, such that medicated feeds are often employed for treatment purposes. Two antimicrobials are approved for use in aquaculture in Denmark: oxolinic acid (OA) and sulfadiazin/trimetoprim (Ministeriet for Fødevarer, Landbrug og Fiskeri, 1997). OA has been demonstrated to be effective against several bacterial pathogens including red mouth disease (Archimbault et al., 1988), and furunculosis (Austin et al., 1983). The pure form of OA is hydrophobic (Samuelsen et al., 1992), such that it is generally delivered via feeding.

Most aquaculture recirculation systems are equipped with biological filters for conversion of total ammonia nitrogen (TAN), excreted by the cultured animals, to less toxic nitrate (review: Skjølstrup et al., 1997). Due to the nature of recirculated systems, chemicals added to the tank water will often enter the biofilter. Although antibiotics, including formalin, oxytetracycline and erythromycin, have been tested for their inhibitory effect upon the nitrification process (Collins et al., 1976; Klaver and Matthews, 1994; Heinen et al., 1995), conflicting results have been reported. Nitrifying bacteria are Gram-negative (Stanier et al., 1990) and OA is primarily effective against such organisms (Alderman et al., 1994). Moreover, OA is persistent, with a reported oral bioavailability in the range of 14%–40% (Björklund and Bylund, 1991; Hustvedt et al., 1991). Accordingly, between 60%–86% of feed-delivered OA will enter the water column either as a solute or complexes. Limited information is available upon the dynamics of OA, derived from medicated feeds, within recirculation systems; or its impact upon nitrification processes.

This investigation was designed to examine the effect of food-derived OA upon the performance of a fluidised bed (FB) biofilter with respect to nitrification rates. In addition, the dynamics of OA in the water column, sludge, feed and fish were evaluated. Animals employed in the experiments were rainbow trout held in an experimental system with a water volume of 1.5 m³. The recirculation system consisted of: FB biofiltration, degassing, cooling and oxygenation components and a novel collection device for sampling of settable particulates (Skjølstrup and McLean, 1997).

2. Materials and methods

2.1. Experimental strategy

FB biofilter performance was evaluated by monitoring daily nitrification rates (DNR), concentrations of ammonia and nitrite concentrations in the FB inlet and outlet water (Table 1). DNR was quantified by examining TAN removal within the main system. Maximum nitrification rate (MNR) was investigated using a scale model containing
biofilter media from the main system. The media in the scale model was provided with ammonia, oxygen and bicarbonate to excess. Heterotrophic activity in the main system was evaluated using on-line analyses of oxygen consumption within the FB.

On the last day of the experimental period, the acute inhibitory level (AIL) of OA on the nitrifying capacity of the FB was also investigated using the scale model. Samples of media from the FB were exposed to different concentrations of OA during MNR determinations. Preliminary MNR runs acted as reference values for comparison. Apart from determination of MNR and nitrite accumulation, AIL runs incorporated water turbidity measurements and bulk water bacteria enumeration. These studies were undertaken because stressed biofilters are characterised by biofilm sloughing/detachment (Characklis and Marshall, 1990). Since handling of FB biofilter media samples was expected to affect biofilm stability, three standard MNR runs were undertaken for each analysis. Thus, in the current investigation two types of FB OA tolerance were investigated, viz. tolerance under typical conditions during OA treatment of rainbow trout in a recirculated system and a tolerance during short-term exposure to high levels of OA in a scale model.

A secondary goal of this research was to examine the fate of feed delivered OA. A mass balance over the experimental period (21 days) was used. It was assumed that the mass balance included five significant contributions. One positive: OA delivered with the medicated feed and four negative: dissolved OA removed during daily water exchange, settleable OA removed during daily sludge discharge, OA accumulation in fish and, finally, unaccounted OA, e.g., losses due to metabolism/degradation, etc. Sample takes for OA analyses are summarised in Table 1. Sludge production was quantified using sludge nitrogen content.

The main system was checked for steady-state conditions for a 7-day period (days -7 to -1) prior to initiation of medication (day 0), which was completed on day 6. Medication was followed by 2 weeks of standard feeding (days 7–21). The experimental period, therefore, extended over 4 weeks, from day-7 through to day 21. Table 1 summarises the schedule for selected parameters sampled throughout the study. Bulk inlet and outlet FB pH, TAN and nitrite measurements were undertaken daily. Temperature and oxygen were monitored on-line.
2.2. The recirculation system

The main recirculation system contained 1.5 m³ of water (Fig. 1) and was a modification of a modular design (Skjølstrup and McLean, 1997), consisting of two inter-connected identical units, each equipped with a fish tank, FB biofiltration, hydraulic sludge separation, oxygenation, degassing and temperature control. Since OA is photo-degradable (Lunestad et al., 1995) the UV-disinfection system incorporated into the original design, was removed (Fig. 1). In the common degasser, water was continuously mixed between the two systems. This method was chosen to guarantee identical inlet water characteristics for the two FBs. FB biofilter performance was,
therefore, evaluated using duplicate observations. In contrast, data on other system components (fish, sludge, OA mass balance), were derived from mixed and homogenized samples from both units.

A special characteristic of both FB biofilters was internal water circulation, which results in prolonged residence times and improved determination of activity rates (DNR and oxygen consumption) due to larger concentration gradients (Skjølstrup and McLean, 1997). FB media consisted of glass weighted PVC (Sadolax I/S, Hobro, Denmark), with a material density of approximately 1.3 kg dry weight dm$^{-3}$. The media had a surface area:volume ratio of 1000 m$^2$ m$^{-3}$ (assuming a smooth surface) at a bed porosity/pore volume of approximately 35%. Bed expansion was 50%, with a hydraulic surface (= cross-section of biofilter) loading of 100 m$^3$ m$^{-2}$ h$^{-1}$. Total surface area within each FB was 14.5 m$^2$, including inner-walls of pipes and tubing, etc.

Oxygen concentrations at the outlet of both FBs were 9.8 ± 1.0 mg O$_2$ l$^{-1}$. Temperature was 16.8 ± 0.3°C and 17.1 ± 0.3°C, respectively. Decreases in pH values due to nitrifying activity were controlled with addition of sodium bicarbonate. The recirculation rate for the whole experimental set-up was 90 ± 1%, i.e., 10 ± 1% of the total water volume in the system was replaced from the domestic supply each day (~ 150 l day$^{-1}$).

Batch experiments on biofilter media from the two FBs were performed using a scale model (Fig. 2). System characteristics such as flow, bed expansion, and temperature, were maintained at identical values to those of the main system. Including biofilter media, total volume of the system was 4.3 l. Temperature, oxygen and pH were controlled manually (Fig. 2), by heat exchange, addition of pure oxygen and sodium bicarbonate, respectively.

Fig. 2. Diagram of the scale model used during batch experiments. (A) FB biofilter media. (B) Oxygen diffuser. (C) Reactor. (D) Valve. (E) Heat exchanger. (F) Pump. (G) pH, oxygen and temperature monitoring.
2.3. Fish and husbandry

Each tank (Fig. 1) was stocked with 20 kg of healthy rainbow trout (Oncorhynchus mykiss). Fish were individually weighed 7 days prior to medication (mean weight for all fish 375 ± 65 g). At study termination on day 21, animals were re-weighed (416 ± 95 g). The fish were fed by automated feeders (100 g tank⁻¹ day⁻¹) throughout the experimental period. This corresponded to approximately 0.5% body weight per day. A commercial high-energy diet (Ecolife 19, BioMar, Brande, Denmark) was used except for the period of medication where a commercially available medicated feed (Aquavet OA, BioMar, Brande, Denmark) was employed instead. The OA content of the medicated feed was 2.35 ± 0.01 mg OA g⁻¹ feed. Oxygen concentration in the two tanks ranged between 7.9 ± 0.4 and 7.8 ± 0.4 mg O₂ l⁻¹. Average temperature for both tanks was 15.3 ± 0.5°C.

2.4. Analytical methods

Temperature in the tanks and biofilters, and oxygen in the biofilter inlet and outlet were monitored on-line by OxyGuard® (OxyGuard, Birkerød, Denmark) combi-probes attached to a control system (see Fig. 1). Readings were recorded for each parameter every 15 min and logged using a Grant® datalogger. Biofilter residence time was calculated using readings from flow meters attached to the two biofilter inlets (SED®, model 785-2-1-315; Granzow, Glostrup, Denmark; Fig. 1). pH was measured daily by Hamilton liquid-glass electrodes connected to a CyperScan pH-meter (Gravquick, Glostrup, Denmark). Biofilter inlet and outlet TAN and nitrite concentrations were measured daily, spectrophotometrically, using Hach® reagents on a Hach DR-2000 spectrophotometer. Sampling for TAN and nitrite determination in the main system was done in duplicate. Water samples from FB inlets and outlets, together with samples of accumulated sludge, were taken in the morning before feeding. System nitrate was measured on 45 μm pre-filtered samples by a Technicon® Traacs 800™ auto-analyzer on samples from the FB biofilter outlets. Water opacity was examined using a Perkin Elmer UV/VIS spectrophotometer (650 nm and 800 nm). Total sludge nitrogen (Kjeldahl-method), was determined in duplicate according to AOAC (1984).

TAN measurement in the scale model, during MNR determination, was based upon single water samples. FB biofilter media for MNR determination were sampled at random times from the main system. Estimates of water column bacteria in the scale model during AIL runs were based on direct counting of cells stained with the fluorochrome, DAPI (4′,6-diamidino-2-phenylidole). DAPI was added to 2 ml of sample to a final concentration of 25 μg DAPI/ml. After 5 min of staining, sampled bacteria were filtered onto a poly-carbonate filter of 0.22-μm pore size and counted by fluorescence microscopy.

OA was measured by HPLC/fluorescence detection for fish, water and feed samples. The analytical method developed by Schneider et al. (1999) was used for all samples although sample preparation varied. Four fish, two from each tank, were sampled before medication and 2 weeks after medication ceased (day 0 and 21, respectively). The whole fish (including flesh, skin and skeleton) were ground and homogenized. Table 1
summarises the times of sludge and water sampling. Sludge OA was measured in combined homogenized samples. Extraction of OA from fish and sludge samples followed the methods described by Schneider et al. (1999). Water samples were analysed without extraction. Feed samples for OA analysis were prepared in the same manner as fish. The HPLC (HP 1100) employed a Phenomenex C18 column held at 28°C and Hitachi FL-7480 detector. Run time was 5 min for an injection volume of 20 µl, with 0.02 M phosphorous acid–acetonitrile–tetrahydrofuran solution as the mobile phase. The analytical method had a limit of quantification of 4 µg OA l⁻¹ (Schneider et al., 1999).

2.5. DNR, MNR and AIL measurements

DNRs within the main system were calculated using inlet and outlet concentrations of TAN, residence time and data relating to total surface area within each FB. MNRs were measured using the scale model (Fig. 2). Samples (559.7 ± 26.2 g DW, n = 36) of biofilter media, together with bulk water, were taken from each of the FBs according to the schedule described in Table 1. All MNR runs were made in duplicate using media sampled from each FB. MNR runs lasted for 180 min with TAN determinations every 30 min. Time 0 and 180 min samples were also analyzed for nitrite. The sampled media were returned to their original biofilter immediately after each run in order to maintain main system media volume. Media used for AIL determination were discarded.

Based upon results from preliminary experiments, oxygen and pH were controlled at intervals of 10.0 to 12.0 mg O₂ l⁻¹ and 6.9 to 7.5, respectively, and an ammonium chloride solution was employed to provide initial TAN concentrations of 6 mg NH₄-N l⁻¹, thereby resulting in unlimited nitrification.

On day 21, AIL runs were undertaken on samples from each FB exposed to various concentrations of OA. Reference values for water column bacteria and absorbance were derived from standard MNR runs undertaken on days 15, 18 and 21. During AIL determinations, absorbance, and the number of water column bacteria were determined for initial and terminal sampling points. The same samples were also analysed for nitrite at the experiment start and end, and for TAN determinations every 30 min. AIL was examined at four concentrations of OA (Sigma, St. Louis, USA): 0.1, 1.0, 10, and 100 mg OA l⁻¹. The OA was dissolved in a 0.5 M NaOH-solution.

3. Results

3.1. Performance of FB biofilters

Concentrations of TAN and nitrite of inlet and outlet water from the FBs are depicted in Fig. 3. Following addition of OA to the water column, inlet TAN and nitrite levels showed sustained decrease throughout the experimental period, with inlet TAN concentrations declining from 0.6 mg NH₄-N l⁻¹ to 0.4 mg NH₄-N l⁻¹ (Fig. 3). In contrast, outlet TAN levels remained relatively constant, thereby indicating decreased nitrification rates (Fig. 3). Inlet nitrite also showed a decrease in concentration, but of a smaller
Fig. 3. Concentrations of TAN and nitrite in the FB, and biofilter inlet and outlet of the experimental system (Fig. 1) over a period of 29 days. Medicated feed was introduced into the system at day 0 and ceased on day 6. Each data point is presented as ± SD, n = 2.

magnitude than that observed for TAN. Both inlet and outlet nitrite was at all times below 0.15 mg NO₂-N l⁻¹ (Fig. 3). Nitrate was constant at 55 ± 4 mg NO₃-N l⁻¹ throughout the period of investigation.

Fig. 4. Consumed oxygen, MNR and DNR for FB biofilters. MNR data were acquired from scale model studies (Fig. 2). See legend to Fig. 3 for further details.
Oxygen consumption within the FB biofilters, representing total aerobic microbial activity, decreased from a mean value of 11.3 g O₂ day⁻¹ prior to addition of OA, to 9.4 g O₂ day⁻¹ (Fig. 4). MNR for sampled biofilter media, together with DNR at the time of sampling is also depicted in Fig. 4. MNR of 0.35 g NH₄-N m⁻² day⁻¹ were recorded for the 7-day period prior to use of medicated feed. Thereafter, MNR declined steadily, achieving levels of 0.25 g NH₄-N m⁻² day⁻¹ by trial end (Fig. 4). Observations upon DNR mimicked those for MNR, although rates were lower (0.05–0.10 g NH₄-N m⁻² day⁻¹; Fig. 4). The range of nitrite accumulation during MNR runs fell between −0.071 and 0.019 mg NO₂-N l⁻¹ (negative values reflecting a reduction from initial concentrations).

### 3.2. Determination of AIL

OA was without effect upon MNR (Fig. 5) and nitrite accumulation (range: −0.058 to 0.005 mg NO₂-N l⁻¹). Similar profiles were recorded for both turbidity and water column bacteria between 0–10 mg OA l⁻¹; however, at a concentration of 100 mg OA l⁻¹ turbidity and water column bacteria increased (Fig. 5). The water changed from clear to a yellow/brownish colour at the highest concentration of OA employed.

![Fig. 5. Scale model derived MNR data for biofilter media exposed to five different concentrations of OA. Also illustrated are the increase in water absorbance and number of suspended bacteria for each experimental run.](image-url)
3.3. The fate of OA

Results from the analysis of OA and nitrogen in both water and sludge samples are illustrated in Fig. 6. Prior to application of medicated feeds, OA was below the level of detection within the experimental system. The daily amount of OA in the sludge reached a peak value (64 mg OA day\(^{-1}\)) 5 days after feeding medication began (Fig. 6). The accumulation of OA in the sludge was triphasic. There was an initial rapid accumulation between 0 and 5 days, followed by a rapid decline between days 7 and 12, and a plateauing until the end of the experiment (Fig. 6). In marked contrast to sludge OA, bulk water concentrations of the antibiotic peaked at day 9 (1.24 mg OA l\(^{-1}\)), 2–3 days after medication ceased. Total sludge nitrogen ranged between 611 and 944 mg N day\(^{-1}\).

### Table 2

<table>
<thead>
<tr>
<th>Fraction</th>
<th>OA (mg)</th>
<th>OA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td>3286</td>
<td>100</td>
</tr>
<tr>
<td>Accumulated in fish (day 21 data)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sludge</td>
<td>520</td>
<td>16</td>
</tr>
<tr>
<td>Waste water</td>
<td>1836</td>
<td>56</td>
</tr>
<tr>
<td>Unaccounted</td>
<td>929</td>
<td>28</td>
</tr>
</tbody>
</table>

Fig. 6. Total nitrogen (N) and OA in accumulated sludge and concentration of OA in bulk water. For further details, see legend to Fig. 3.
with highest values being recorded during the earlier parts of the experiment, and lower levels being observed following a period of medication (Fig. 6).

Analysis of sludge and bulk water (equivalent to the system outlet concentration) OA provided data for estimation of system mass balance (Table 2). The amount of OA detected in fish at day 21 of the trial represented a minor component of the mass balance (<0.1% of total). In contrast, 56% was removed from the system via water exchange, 15% with settleable particulates, leaving approximately 28% unaccounted for by the analyses.

4. Discussion

In this study, and under practical conditions (at a stocking density of 25 kg m\(^{-3}\) system water), feed-based OA had no effect upon nitrification rates of a system based upon FB biofilters, at the dosage employed (fed 0.5% body weight day\(^{-1}\) with feed containing 0.235% w/w OA). Nevertheless, OA, derived from trout effluents has been shown to retain a broad-spectrum activity on several different strains of sediment and water column bacteria (Spanggaard et al., 1993). Other antibacterial agents, including erythromycin (Collins et al., 1976) and oxytetracycline (Klaver and Matthews, 1994), have been shown to inhibit biofilter nitrification rates.

The conclusion that OA was without impact upon the biofilter’s nitrification rates appears, at first glance, to be inconsistent with the presented results (Fig. 4), since a general decrease was registered for MNR, DNR and total oxygen consumption following fish treatment with medicated food. This effect is likely to be due to decreased microbial activity within the FB, which could be interpreted as a direct inhibition by OA. However, it is important to note that recirculation systems continually evolve with respect to biofilm kinetics. In a system such as the one used here, the state of biofilm transformation is likely to be even more pronounced due to the relatively high surface area to water ratio (tubes, pipes, tank and degasser walls, etc.) when compared to larger systems. Clearly, the experimental system provides ample opportunity for colonization and subsequent growth of nitrifying biomass outside the FB. It is noteworthy that nitrate added to the system via feeding and set-up water, was maintained at a constant level throughout the experimental period. Moreover, sludge nitrogen did not increase (Fig. 6) as a result of, for example, reduced appetite (uneaten feed pellets) among the stocked animals. If OA were to have influenced the efficiency of the FB, then concentrations of TAN in the inlet and outlet water would have increased. In fact, the opposite was observed (Fig. 3). Clearly, the results indicate an increase in total ammonia removal that in all likelihood occurred due to nitrifying bacteria biomass build up within the system as a whole.

Several studies have reported reduced efficacy of antibiotics for biofilm bacteria (review: Gandar, 1996). This may occur due to biofilm resistance to biocides. Since AIL runs were performed on biofilter media that had experienced earlier exposure to OA, the occurrence of acclimation (resistance) cannot be discounted. There exists the possibility, therefore, that the lower limit for an OA-induced effect upon biofilter performance may occur at lower concentrations than indicated by the scale model studies. Biofilm
resistance to antibiotics may also be enhanced due to the protective nature of the exopolysaccharide matrix, which envelopes biofilm cells (Gandar, 1996). However, while this effect can not be excluded from the present study, one advantage of FBs, compared to other designs, relates to the steady conditioning of the system resulting in a thinner biofilm (Ruggeri et al., 1994) and hence reduced barrier potential. Alternatively, it has been hypothesised that the electrical charge of the polysaccharide chains might influence antimicrobial diffusion (Gandar, 1996).

Comparison of OA concentrations reached in the bulk water of the experimental system with those for the selected concentrations during AIL determination, revealed that only the two highest OA concentrations tested (10 and 100 mg OA l⁻¹) returned elevated values. Since no effects were observed during long-term exposure of the model system to OA, acute inhibition of MNR determination would not be expected in the scaled system for concentrations of 0.1 and 1 mg OA l⁻¹. In fact, MNR remained unaffected even at the highest concentration of OA tested. The increase in water column bacteria and the development of a yellow/brown colour during scale model tests performed at 100 mg OA l⁻¹, are believed to be a result of dissolution of the biofilm. Such a phenomenon is a result of an indisposed (stressed) biofilm. It is credible that continued sloughing of biofilm from the FB within the main system might lead to a reduced nitrifying capacity over extended periods but this remains conjecture. The mechanisms underlying the apparent lack of effect for OA needs to be explained since biocides in general have been suggested as being a major cause for the sudden detachment of biofilm (sloughing) (Characklis and Marshall, 1990). A disadvantage of the experimental strategy employed was that sampled biofilter media had been exposed to OA for varying durations such that the possibility remains that the microflora adapted to OA exposure.

Although nitrification was unaffected in the main system over the period of investigation, there is reason to believe that the performance of a nitrifying biofilter would be influenced by an OA concentration \( \geq 100 \) mg OA l⁻¹, as indicated by the scale model studies. The concentration of OA in the bulk water is obviously a function of fish density. A fish density of 2.5% (kg wet weight of fish kg⁻¹ tank water × 100) was used in this experiment. Densities of up to 20% have been reported for some commercial systems. Thus, five times higher levels of bulk water OA might, in theory, be achieved in such systems were the identical doses of OA to be used.

Most of the OA added via the feed left the system either in solute (water exchange) form or associated with particulates (sludge removal), with limited amounts being accounted for by fish tissues at day 21. Direct determination of OA bioavailability for the fish was not examined. However, there was a delay of 2 days following cessation of medication before the peak OA levels occurred in the water column. The uptake and excretion or OA degradation by the fish was, therefore, likely to be much higher than the analytical results on fish samples indicate. The precise method by which OA enters the water column from the feed remains to be determined. However, the possibility that some of the food-based OA entered the water directly from the feed pellet cannot be discounted. Since 72% of the OA delivered to the system was “recovered”, 28% remains “undefined”. With bioavailability of OA reported in the range of 14%–40% (Björklund and Bylund, 1991; Hustvedt et al., 1991), it is likely that most of the OA
absorbed by the fish was biotransformed and metabolized during the experimental period (see Ishida, 1992). In addition, OA would have degraded over time, throughout the entire system (see Lunestad et al., 1995). The data with respect to OA thus represent a mass balance for the system as a whole. It is noteworthy that the sludge production appeared to decline following OA treatment. This might have occurred because of increased soluble production due to alterations in food processing and faecal stability. Future studies upon the possible effect of OA upon FB performance should be undertaken using higher concentrations than employed in the present study and for extended periods. Attention should be leveled at the stability and kinetics of system biofilms following antibiotic exposures.

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