

**QUANTITATIVE HPLC DETERMINATION  
AND PRELIMINARY ASSESSMENT OF DISSOLVED,  
SUSPENDED, AND SETTLED OXOLINIC ACID  
FROM TURBOT FARM**

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**ABSTRACT**

Quantification of outputs of antibacterial agents from fish farms is necessary to assess their environmental impact. In effluents, antibacterial agents are dissolved or associated with mineral or organic materials, the heaviest of which quickly settle at the exit of the fish farm. Oxolinic acid (OA) is one of the quinolones the most frequently used in turbot farms. For the first time, procedures are described for determination of dissolved, suspended, and settled forms of OA by reversed-phase high-performance liquid chromatography. Chromatography was performed on a 5  $\mu\text{m}$  C<sub>18</sub> cartridge, with a mixture of acetonitrile and aqueous orthophosphoric acid solution as mobile phase, and fluorescence detection. OA was easily and completely isolated by solid–liquid extraction. The method is selective, accurate, and precise; limits of quantitation were 0.005  $\mu\text{g mL}^{-1}$ , 0.025  $\mu\text{g}$  per filter, and 0.025  $\mu\text{g g}^{-1}$ , respectively for dissolved, suspended, and settled forms of OA. These methods enabled initial assessment of the amounts of dissolved, suspended, and settled forms of OA (72, 23, and 2%, respectively) of the antibacterial agent administered.

**INTRODUCTION**

Because of its high potency against Gram-negative bacteria, oxolinic acid (OA) is used to combat bacterial infections in fish farming. The drug is usually administered to fish mixed with feed at a dosage rate of 10–20  $\text{mg kg}^{-1}$  biomass  $\text{day}^{-1}$  for 8–10 days. It is becoming apparent that a large fraction of the OA administered to the fish, typically as medicated

feed, is not absorbed or retained by the animal but is released to the environment by three routes [1–4]. First, some of the medicated feed supplied is not ingested and, instead, falls directly to the bottom of the pond. Second, some of the OA is not absorbed during passage through the gut and is released to the environment via the faeces. Finally, some of the absorbed OA is excreted via the urine and bile in an unmetabolized and microbiologically active form.

Effluents from fish culture may contain OA in any of three forms [1,5–7]. First, OA may be present dissolved in water. Second, because water contains particles in suspension (mineral and organic matter, feed, faeces, ...), OA may also be present suspended in water. Finally, because of deposition of feed and faecal matter in aquaculture ponds, OA may also be present in settled matter. The amounts of OA in the effluent could be of environmental significance if they affect microbial communities; this may affect the viability of continued fish culture if it alters the rate of degradation of organic matter or promotes the proliferation of antibacterial-resistant strains of pathogenic bacteria.

One aspect of an investigation into the amount of OA output from turbot (*Scophthalmus maximus*) culture during and after chemotherapy is reported in this paper. This study required the development of analytical methods for determination of OA in suspended and settled matter, because none was described in the literature. One objective of the study was, therefore, to develop precise and accurate HPLC methods for determination of dissolved, suspended, and settled forms of OA, to enable experimental quantification of OA output in different forms from a land-based turbot farm.

## EXPERIMENTAL

### Chemicals, Reagents, and Solutions

Standards of the antibiotics oxolinic acid (OA) and nalidixic acid (NA; used as internal standard), both 98% purity, were obtained from Sigma (St Louis, MO, USA).

Stock standard solutions containing 1,000  $\mu\text{g mL}^{-1}$  OA or NA were prepared by accurately weighing 50.0 mg of the antibiotics (Mettler, Greifensee, Switzerland, AE 240 balance capable of weighing to  $\pm 0.0001$  g) in separate 50-mL volumetric flasks, dissolving in 0.03 mol L<sup>-1</sup> sodium hydroxide solution, and diluting to volume with the same solution. The

solutions were stored at 4°C for one month. Chromatographic standard solutions were prepared freshly every day by appropriate dilution of the stock standard solutions with LC mobile phase. Solutions for calibration plots and quality control were prepared freshly every day by appropriate dilution of the stock standard solutions with water.

Adjustable 5 mL pipettes (Biohit, Helsinki, Finland) and microadjustable 20-1000 µL pipettes (Labsystem, Basingstoke, UK) were used for preparation of solutions. Volumetric flasks were class A (Schott, Mainz, Germany).

Acetonitrile and chloroform were HPLC-grade from VWR (Darmstadt, Germany) and orthophosphoric acid was analytical grade, purity 85%, from VWR. Sodium hydroxide was analytical grade and 1 mol L<sup>-1</sup> hydrochloric acid was prediluted analytical grade, both from Panreac Quimica (Barcelona, Spain).

Solutions of sodium hydroxide containing 0.1, and 0.2 mol L<sup>-1</sup> were prepared by dissolving 4 or 8 g sodium hydroxide in water in 1000-mL volumetric flasks, diluting to volume with water, and mixing. A solution of sodium hydroxide containing 0.03 mol L<sup>-1</sup> was prepared by diluting 15 mL 0.2 mol L<sup>-1</sup> sodium hydroxide to 100 mL with water in a volumetric flask, and mixing. Orthophosphoric acid solution (0.02 mol L<sup>-1</sup>) was prepared by diluting 1.348 mL 85% orthophosphoric acid with 1000 mL water in a volumetric flask and mixing.

Water was Ultrapure from Milli Q Academic equipment (Millipore, Bedford, MA, USA).

## **Extraction and Clean-Up Procedures**

### *Dissolved Oxolinic Acid*

Seawater samples (2 mL) were filtered through a Minisart NML cellulose acetate membrane, 26 mm diameter, 0.45 µm porosity (Sartorius AF, Goettingen, Germany) and the filtrate was transferred to a 2-mL centrifuge tube with a cone-shaped bottom (Sarstedt, Numbrecht, Germany) and centrifuged at 12,000g for 5 min at 4°C with a Jouan (Saint-Herblain, France) MR 1822 centrifuge. The supernatant was then transferred to an HPLC autosampler vial (Gilson 1-mL vials for automatic injector).

### *Suspended Oxolinic Acid*

A Pall Gelman (Ann Arbor, MI, USA) A/E cellulose acetate membrane, 47 mm diameter, 1.0 µm porosity was dried at 60°C for 18 h and

then weighed. A seawater sample (100 mL) was filtered through the membrane by means of an HPLC Sartorius (Goettingen, Germany) filtration unit. The membrane was again dried at 60°C for 18 h then reweighed. The membrane was frozen at -18°C for at least 24 h and then placed in the receptacle of a Virtis (Gardiner, NY, USA) S23 cutter homogenizer. Aqueous NA solution (10 µg mL<sup>-1</sup>, 100 µL) and sodium hydroxide solution (0.2 mol L<sup>-1</sup>, 30 mL) were added and the mixture was homogenized for 2 min. The suspension obtained was transferred to a 50-mL centrifuge tube with a cone-shaped bottom. The receptacle of the cutter homogenizer was rinsed again with sodium hydroxide solution (0.2 mol L<sup>-1</sup>, 20 mL), the combined extracts were centrifuged at 12,000g for 20 min at 4°C, and the supernatant was transferred into a 250-mL decantation flask. The tube was again centrifuged at 12,000g for 5 min at 4°C. Hydrochloric acid (1 mol L<sup>-1</sup>, 15 mL) and chloroform (5 mL) were added to the decantation flask containing the combined supernatant, the mixture was homogenized for 2 min, and the chloroform layer was transferred to a 16-mL glass tube (TVU16; CML, Nemours, France). The extraction was repeated twice with 3 mL then 1 mL chloroform, the combined chloroform extracts were centrifuged at 12,000 g for 5 min at 4°C, and the supernatant was evaporated to dryness in a 16 mL glass tube under a nitrogen stream at 40°C (Ibbisch evaporator; Bioblock Scientific, Illkirch, France). The dry residue was dissolved in 0.5 mL mobile phase and vortex mixed (Top-Mix II; Heidolph). The extract was then transferred to an HPLC autosampler vial.

#### *Settled Oxolinic Acid*

Settled matter (1.0 g) was accurately weighed into a 15-mL centrifuge tube with a cone-shaped bottom and aqueous NA (internal standard) solution (10 µg mL<sup>-1</sup>, 100 µL) and sodium hydroxide solution (0.1 mol L<sup>-1</sup>, 4 mL) were added. The mixture was homogenized for 10 min with a Reax 2 (Heidolph, Kelheim, Germany) agitator and centrifuged at 4,000 g for 10 min at 4°C. The supernatant was transferred to a 15-mL centrifuge tube with a cone-shaped bottom by means of a transfer pipette and the extraction was repeated once with sodium hydroxide solution (0.1 mol L<sup>-1</sup>, 4 mL). Hydrochloric acid (1 mol L<sup>-1</sup>, 3 mL) and chloroform (4 mL) were added to the centrifuge tube containing the combined supernatant. The mixture was homogenized for 10 min with the agitator and then centrifuged at 4,000g for 10 min at 4°C. The chloroform extract was transferred to a 16 mL glass tube and the extraction was repeated once with 4 mL chloroform. The combined chloroform extracts were evaporated to dryness un-

der a stream of nitrogen at 40°C and the dry residue was dissolved in 1.0 mL mobile phase and vortex mixed. The supernatant was then transferred to an HPLC autosampler vial.

### **Preparation and Analysis of Fortified Samples**

Fortified samples were prepared at least 1 h before the beginning of the extraction and clean-up procedures.

#### *Dissolved Oxolinic Acid*

Calibration plots were established by spiking 9.9 mL drug-free seawater samples with 100  $\mu\text{L}$  water or aqueous OA solutions (0, 0.5, 5, 50, 100, or 200  $\mu\text{g mL}^{-1}$ ) to yield 0, 0.005, 0.05, 0.5, 1, and 2  $\mu\text{g mL}^{-1}$  seawater. Quality-control standards (0.05, 0.15, and 0.8  $\mu\text{g mL}^{-1}$  seawater) were prepared in the same way as the calibration solutions except that aqueous OA solutions were prepared by another person from another OA stock standard solution.

#### *Suspended Oxolinic Acid*

Calibration plots were established by spiking drug-free membrane samples containing suspended matter (mean 37 mg) with 100  $\mu\text{L}$  water or aqueous OA solutions to yield 0, 0.025, 0.05, 0.25, 0.75, and 1  $\mu\text{g}$  per membrane. Quality-control standards (0.025, 0.5, and 1  $\mu\text{g}$  per membrane) were prepared in the same way as the calibration solutions except that aqueous OA solutions were prepared by another person from another OA stock standard solution.

#### *Settled Oxolinic Acid*

Calibration plots were established by spiking drug-free settled matter with 100  $\mu\text{L}$  water or aqueous OA solutions to yield 0, 0.025, 0.1, 0.5, 1, and 2  $\mu\text{g g}^{-1}$ . Quality-control standards (0.05, 0.5, and 1.5  $\mu\text{g g}^{-1}$ ) were prepared in the same way as the calibration solutions except that aqueous OA solutions were prepared by another person from another OA stock standard solution.

### **Chromatography**

HPLC was performed with an isocratic pump and an automatic injector with a 10- $\mu\text{L}$  loop (307 pump and 234 auto-injector; Gilson, Villiers Le Bel, France), cartridge oven (CTO.10AS VP; Shimadzu, Kyoto, Japan), fluorescence detector (FP-1520; Jasco, Tokyo, Japan), integrator, and com-

puter-assisted data capture and analysis system (D 2500 integrator and HPLC Manager software system; VWR). Compounds were separated on a 125 mm × 4 mm i.d., 5 µm particle size, PuroSpher 180 RP-18 E cartridge equipped with a 4 mm × 4 mm i.d. guard cartridge containing the same packing material (both from VWR). The mobile phase was prepared by mixing 670 mL 0.02 mol L<sup>-1</sup> orthophosphoric acid solution with 330 mL acetonitrile.

The injection volume was 10 µL, the flow rate was 0.8 mL min<sup>-1</sup>, and the cartridge oven temperature was 27°C. Fluorescence detection was performed with an excitation wavelength of 325 nm and an emission wavelength of 365 nm. The run time was 15 min with 3 min equilibration between runs. After each day of operation the analytical and guard cartridges were flushed for 50 min with a 50:50 (v/v) mixture of acetonitrile and water at a flow rate of 0.5 mL min<sup>-1</sup>. The guard cartridge was exchanged at intervals of 1000 sample injections.

### Validation

For dissolved forms of OA calibration plots were drawn by plotting peak heights of OA (µV) against known concentrations of OA (µg mL<sup>-1</sup>). For suspended and settled forms of OA, calibration plots were drawn by plotting the OA-to-NA peak-height ratio against the OA-to-NA quantity or concentration ratios. Linearity, regression, and extraction recoveries were evaluated by extracting and analyzing one replicate per fortification level on each of four days. Linearity and regression were studied by calculating the slope, intercept, and *F*-ratio of the ANOVA and the correlation coefficient. The bias was also calculated for each fortification by use of the equation:

$$\text{Bias} = [(C_c - C_t)/C_t] \times 100$$

where  $C_c$  is the calculated concentration or quantity of the analyte (µg mL<sup>-1</sup>, µg g<sup>-1</sup> or µg) and  $C_t$  is the theoretical concentration or quantity of the analyte (µg mL<sup>-1</sup>, µg g<sup>-1</sup> or µg). Extraction recovery of OA and NA was calculated by comparing individual peak heights with peak heights obtained from corresponding chromatographic standard solutions, by use of the equation:

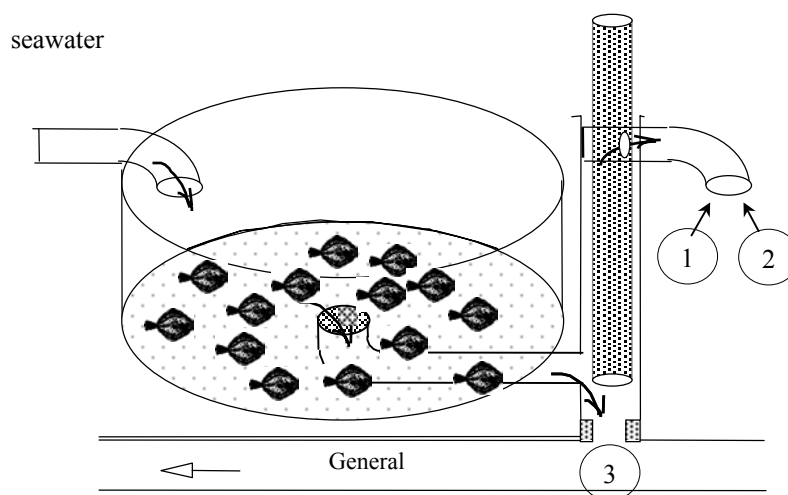
$$\text{Extraction recovery} = [PH_{fs}/PH_{ss}] \times 100$$

where  $PH_{fs}$  is the peak height obtained from a fortified sample and  $PH_{ss}$  is the peak height obtained from a chromatographic standard solution at the

same concentration. Quality-controls samples were used to evaluate accuracy and precision by extracting and analyzing three replicates per fortification level on each of four days. The concentrations or quantities of OA in the quality controls ( $\mu\text{g mL}^{-1}$ ,  $\mu\text{g g}^{-1}$  or  $\mu\text{g}$ ) were calculated from the spiked sample calibration plots. Accuracy was evaluated by calculating the bias as described above. Precision was studied by calculating the relative standard deviations of repeatability and intermediate precision. The limits of detection and quantitation were calculated as the smallest concentrations or quantities giving signal-to-noise ratios  $>3$  and  $>10$ , respectively. For each day of validation the six chromatographic standard solutions, the six calibrators, and the nine quality controls were successively injected into the LC system.

### Fish Device and Sampling in the Fish Farm

One thousand 530-g turbot were kept in a  $10\text{ m}^3$  seawater tank with a continuous flow of seawater (flow rate  $2.5\text{ m}^3\text{ h}$ , temperature  $8.6\text{--}13.9^\circ\text{C}$ , suspended matter  $17.6\text{--}44.0\text{ mg L}^{-1}$ ). They were fed three times per day with OA-medicated pellets for 10 days (dose  $36\text{ mg kg}^{-1}$  fish weight  $\text{day}^{-1}$  on the first two days,  $24\text{ mg kg}^{-1}$  fish weight  $\text{day}^{-1}$  for eight days). Seawater, suspended matter, and settled matter from the fish tank were sampled manually (Fig. 1) every three hours in a daily survey and once a day in a 15-day survey.



**Fig. 1**

Farm tank system and location of sampling points

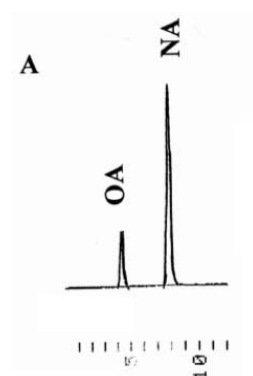
For dissolved OA, seawater was sampled directly in the outflow and placed in 1.5 mL Eppendorf tubes. For suspended OA, 0.5 L seawater sampled in the outflow was filtered through a 1.2  $\mu\text{m}$  pore Whatman GF/C filter in accordance with the method developed by Aminot et Chaussepied (1983) for suspended material. After filtration, membranes were individually placed in 150 mL plastic bottles. For settled OA, settled matter was collected in the waste outlet, homogenized, and sampled in 150-mL plastic bottles. Waste was evacuated daily. All samples were kept frozen ( $-20^{\circ}\text{C}$ ) until analysis.

## RESULTS AND DISCUSSION

### Analytical Methods

#### *Chromatographic Conditions*

Reversed-phase HPLC has often been used for determination of quinolone antibiotics and good performance, in terms of plate numbers, has been achieved [8]. In our experiment the PuroSpher 100-RP 18 E pre-packed cartridge resulted in narrow OA and NA peaks and good capacity factors, symmetry factors, theoretical plate counts, and resolution [9]. With the cartridge the best mobile phase was a mixture of an organic modifier, acetonitrile, and a predominantly aqueous eluent, aqueous orthophosphoric



**Fig. 2**

HPLC chromatogram (peak height in  $\mu\text{V}$ , time in min) obtained from a standard solution containing oxolinic acid (OA;  $0.5 \mu\text{g mL}^{-1}$ ) and nalidixic acid (NA;  $1.0 \mu\text{g mL}^{-1}$ ) in LC mobile phase. Conditions: mobile phase, acetonitrile–0.02 M aqueous orthophosphoric acid solution, 33:67 (v/v); cartridge, 125 mm  $\times$  4.6 mm, C18E (5  $\mu\text{m}$ ); flow rate,  $0.8 \text{ mL min}^{-1}$ ; excitation wavelength, 325 nm; emission wavelength, 365 nm; injection volume, 10  $\mu\text{L}$

acid solution [9]. Under these operating conditions, OA and NA were eluted in 4.2 and 7.7 min, respectively (Fig. 2).

### *Extraction and Recovery*

The extraction of OA and NA from suspended and settled matter used, first, sodium hydroxide (OA and NA are soluble in aqueous alkaline solutions), then hydrochloric acid and chloroform (OA and NA are soluble in organic solvents but not in aqueous acid solution). This procedure gave high extraction recoveries with low relative standard deviations (Table I). No extraction was necessary for quantification of dissolved forms of OA. Seawater was merely filtered before centrifugation. Figure 3 shows chromatograms obtained from blank and spiked samples. The resulting extracts were free from interference, indicating that satisfactory purification could be achieved by these methods. The extraction and clean-up procedures were found to be appropriate and were further evaluated for performance.

**Table I**

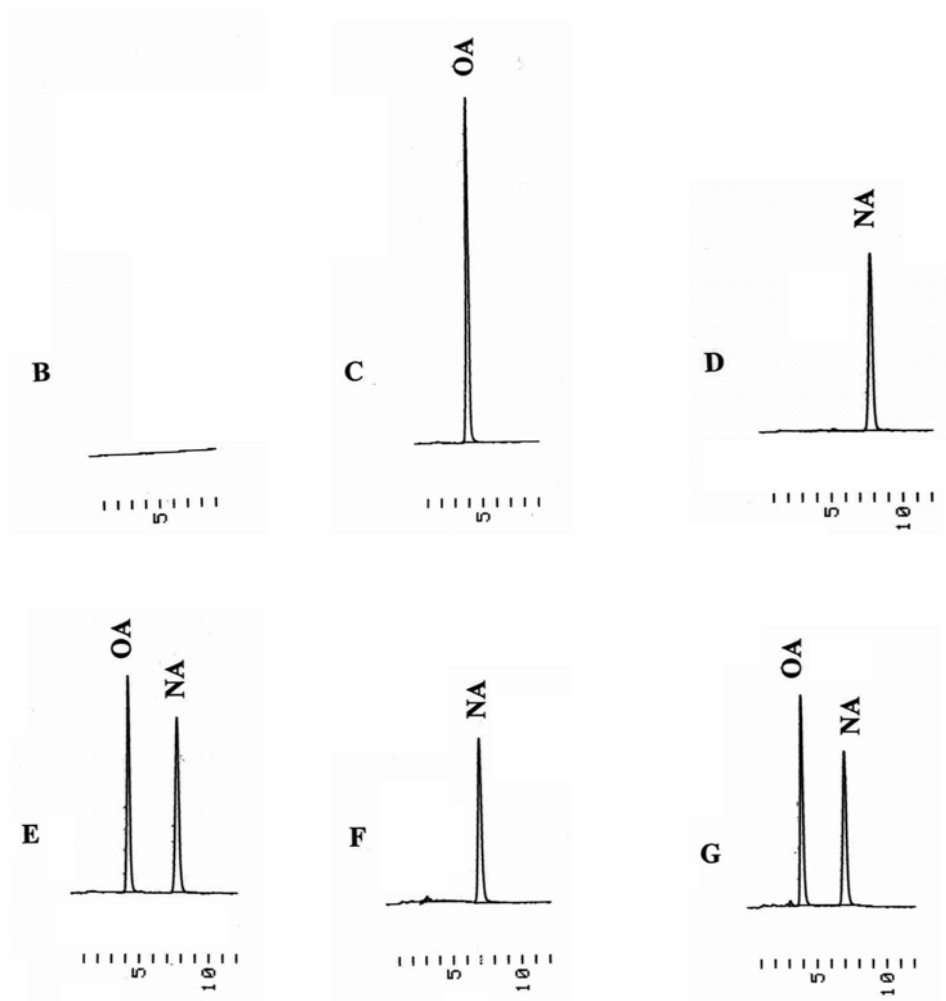
Linearity, regression results, extraction recoveries, limits of detection and quantitation, accuracy and precision: data from validation of the method for determination of dissolved, suspended and settled forms of oxolinic acid (OA)

|   | Dissolved forms of OA         | Suspended forms of OA | Settled forms of OA          |
|---|-------------------------------|-----------------------|------------------------------|
| <i>Linearity and regression</i>                             |                               |                       |                              |
| Concentration range   | 0.005–2 $\mu\text{g mL}^{-1}$ | 0.025–1 $\mu\text{g}$ | 0.025–2 $\mu\text{g g}^{-1}$ |
| Correlation coefficient                                     | $\geq 0.999$                  | $\geq 0.999$          | $\geq 0.999$                 |
| Bias (%) <sup>a</sup>                                       | -3.7 / +11.5                  | -10.3 / +14.3         | -3.5 / +7.1                  |
| <i>Extraction recovery</i>                                  |                               |                       |                              |
| Mean (%)  | –                             | 94.3                  | 98.0                         |
| RSD (%) <sup>b</sup>  | –                             | 1.6                   | 12.9                         |
| Limit of detection  | 0.002 $\mu\text{g mL}^{-1}$   | 0.008 $\mu\text{g}$   | 0.008 $\mu\text{g g}^{-1}$   |
| Limit of quantitation                                       | 0.005 $\mu\text{g mL}^{-1}$   | 0.025 $\mu\text{g}$   | 0.025 $\mu\text{g g}^{-1}$   |
| <i>Accuracy</i>   |                               |                       |                              |
| Bias (%) <sup>a</sup>                                       | -5.2 / +6.0                   | -6.0 / +10.3          | -10.3 / +2.7                 |
| <i>Precision</i>  |                               |                       |                              |
| RSD <sup>c</sup> of repeatability (%) <sup>a</sup>          | 0.3 / 1.6                     | 0.8 / 2.0             | 1.1 / 6.7                    |
| RSD <sup>c</sup> of intermediate precision (%) <sup>a</sup> | 1.4 / 2.7                     | 0.8 / 2.0             | 1.6 / 12.4                   |

<sup>a</sup>Minimum / Maximum

<sup>b</sup>Relative Standard Deviation ( $n = 20$ )

<sup>c</sup>Relative Standard Deviation ( $n = 12$ )



**Fig. 3**

HPLC chromatograms (peak height in  $\mu\text{V}$ , time in min) obtained from: B, blank seawater; C, seawater spiked with  $0.25 \mu\text{g mL}^{-1}$  oxolinic acid (OA); D, blank suspended matter; E, suspended matter spiked with  $0.25 \mu\text{g}$  OA and  $1 \mu\text{g}$  nalidixic acid (NA); F, blank settled matter; G, settled matter spiked with  $0.5 \mu\text{g g}^{-1}$  OA and  $1 \mu\text{g g}^{-1}$  NA. Conditions: mobile phase, acetonitrile– $0.02 \text{ M}$  aqueous orthophosphoric acid solution, 33:67 (v/v); cartridge,  $125 \text{ mm} \times 4.6 \text{ mm}$ , C18E ( $5 \mu\text{m}$ ); flow rate,  $0.8 \text{ mL min}^{-1}$ ; excitation wavelength,  $325 \text{ nm}$ ; emission wavelength,  $365 \text{ nm}$ ; injection volume,  $10 \mu\text{L}$

### *Linearity, Regression, Accuracy, Precision, and Limits of Detection and Quantification*

Linearity and regression studies were performed separately for each calibration plot [10]. The high values of the correlation coefficients ( $\geq 0.999$ ) are indicative of good correlations between OA concentrations or quantities and peak heights (Table I). The  $F$ -values for the linearity tests were higher than the critical value (data not shown) and the bias was between  $-15\%$  and  $+15\%$  for all fortification levels for the calibration plots; linear regressions were, therefore, significant at the 0.00001 level in the concentration ranges investigated (data not shown).

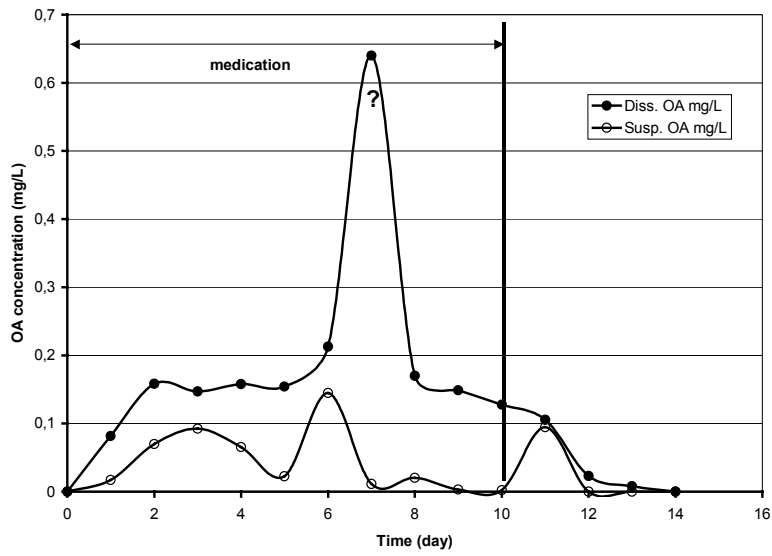
The bias for analysis of the quality-control standards was always between  $-15\%$  and  $+15\%$  for all fortification levels (Table I). The relative standard deviations of repeatability and intermediate precision were less than 15% (Table I). The analytical methods were therefore accurate and precise [10,11].

Limits of detection and quantitation of OA are given in Table I. Limits of quantitation were acceptable (relative standard deviation  $< 15\%$ ,  $n = 20$ ) because the mean values of the drug peak heights were significantly different from the intercepts at the 0.05 level and greater than three standard deviations [10,11]. Limits of quantitation were suitable for environmental monitoring of dissolved, suspended, and settled forms of OA at the concentrations investigated.

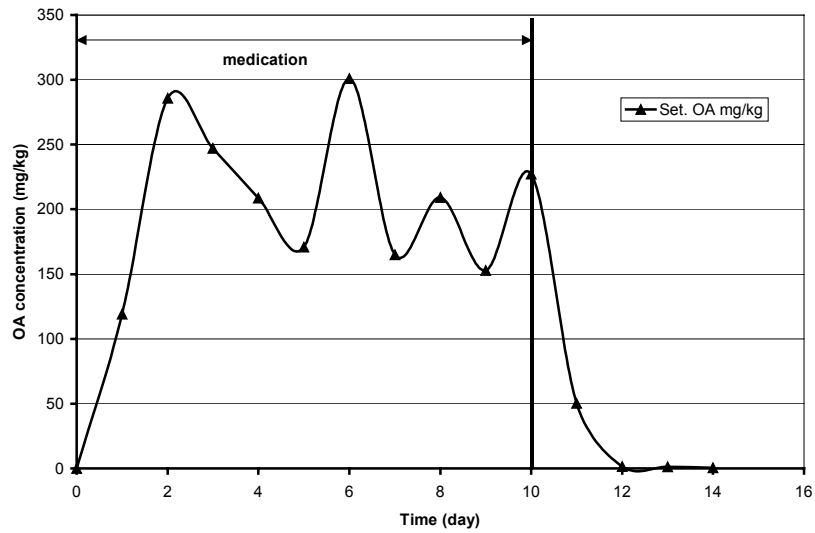
### **Relative Amounts of Dissolved, Suspended, and Settled OA**

#### *Survey of Concentrations of Dissolved, Suspended, and Settled OA*

Dissolved OA concentrations increased until day 2 (Fig. 4), after which concentrations remained more or less constant (between 0.15 and 0.21 mg L<sup>-1</sup>) until day 10, except for an inexplicable peak on day 7. Levels quickly decreased after the end of chemotherapy and became negligible on day 13. Suspended OA concentrations were lower than dissolved OA concentrations and varied substantially during chemotherapy (Fig. 4). Two days after medication, they were below the limit of detection of the method. Settled OA concentrations (Fig. 5) increased until day 2 and then remained between 150 and 300 mg kg<sup>-1</sup>. They rapidly decreased after end of chemotherapy. For the fifteen-day survey, 95% confidence intervals were calculated.



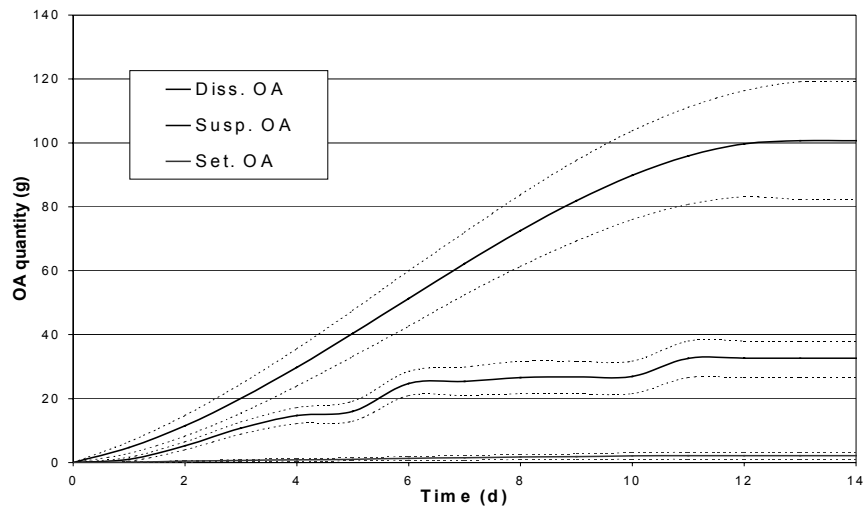
**Fig. 4**  
Survey of dissolved and suspended concentrations of OA



**Fig. 5**  
Survey of settled concentrations of OA

### *Assessment of Quantities of Dissolved, Suspended and Settled OA Released*

We assumed the OA concentration measured on one day was an average of those measured during a cycle on the same day and that seawater renewal rate remained constant throughout all fifteen days of the survey. Daily quantities of dissolved and suspended OA released were calculated by multiplying daily dissolved or suspended OA concentrations by the daily seawater flow volume. Daily quantities of settled OA released were calculated by multiplying the daily settled OA concentrations by the daily mass of waste corresponding to 30% of the feed delivered [12]. Finally, cumulative quantities were calculated for each form of OA. As shown in Fig. 6, a plateau was reached on day 13, on which we believed all release of OA from the treated turbot had ended. The relative proportions of the different forms of OA in the effluent were calculated. The total quantity



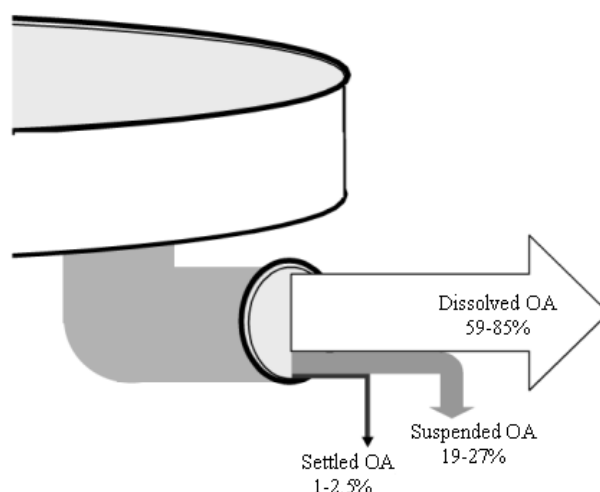
**Fig. 6**

Cumulative amounts of dissolved, suspended, and settled OA released. Dashed lines corresponds to the 95% confidence interval limits

of OA released ranged from 79 to 115% of the quantity administered. The ranges of the percentages of the different forms of OA released relative to the amount of OA administered (Fig. 7) showed that dissolved OA (59–85%) predominated over suspended and settled OA (20–30%).

Because these results were based on assumptions that must be confirmed, they indicate only within an order of magnitude the quantity of OA

released. More precise data are needed. These can be obtained by hourly monitoring of OA concentrations and recording daily seawater outflow and quantities of waste.



**Fig. 7**

Ranges of forms of OA in turbot farm effluent

## CONCLUSION

The analytical methods described enable selective, reliable, accurate, and precise determination of dissolved, suspended, and settled forms of OA. The methods do not require complex extraction or derivatization techniques. An analyst familiar with the methods could easily process eighty samples a day for dissolved forms of OA or twenty samples a day for suspended or settled forms. As assumed on the basis of the liquid nature of turbot faeces, dissolved forms of OA seemed to predominate in turbot effluent.

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