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ORGANIC WASTE AND ANTIBIOTICS FROM AQUACULTURE

by

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ABSTRACT

During the past year three joint projects have been conducted concerning the fate of organic waste and antibiotics from various Norwegian fish farms. The projects are investigating the following topics:

- decomposition rate of organic waste, flux between the sediment and water of various inorganic compounds, and distribution of waste through the marine food chain
- the persistance of antibiotics in sediments and its degradation in seawater
- development of resistance in bacteria in the sediment

The collaboration of the projects is planned to be extended next year into a larger project as part of a national program concerning the ecological effects of antibiotics.

INTRODUCTION

Traditionally, one has not been concerned with environmental effects of mariculture. However, with the growth of the industry, problems such as decreasing productivity emerge after a few years (Braaten et al, 1983). At the same time, the possible environmental effects have become an issue of discussion, and the demand for more knowledge has been increasing. Initially, the main area of interest was the fate of organic matter, waste food and fecal pellets, but lately other topics such as the fate and the effects of antibiotics are being addressed.

Fish farms consisting of net pens give rise to an export of organic waste to the underlying sediment and, in the case of disease treatment, this material will contain various amounts of antibiotics. The organic waste itself can change the sediment dramatically from a mineral-rich, bioturbated, oxic sediment to an organically rich, non-bioturbated, anaerobic one. This again influences the water body by consuming oxygen in high amounts and releasing various compounds like ammonia and hydrogen sulphide. The latter substance, which is very toxic, is often produced at high rates in sediments under fish farms and can be transported into the water column by means of both diffusion and ebullition.

Organic waste containing antibiotics will precipitate to the sea floor where resistance may be developed in the bacterial flora. Antibiotics may dissolve in the pore water and be released to the water column and later accumulate in the wild fauna.

This paper presents three joint projects dealing with the following topics:

- decomposition rate of organic waste, flux between the sediment and water of various inorganic compounds, and distribution of waste through the marine food chain
- the persistance of antibiotics in sediments and its degradation in seawater
- development of resistance in bacteria in the sediment

We present here the preliminary results, since measurements and analyses are still ongoing.

MATERIALS AND METHODS

Sampling Locations

The fate of organic waste was investigated at seven fish farms chosen to represent a gradient in accumulated organic matter and different feed types. Furthermore, a control station with a sandy bottom, away from the farms, was also chosen. All the farms were located in Hordaland County, on the west coast of Norway. Three of the locations were also investigated for bacteria number, resistant bacteria and antibiotics in the sediment. The medication had been administered by the farmers in the course of operations.

The current was weak at all farms, except one, throughout the year, and the annual bottom temperatures varied between 4^{0}C and 12^{0}C. Depths were between 7 and 21 meters with most of the farms located over 12-14 meters, and salinity at the bottom was between 33 - 35 ppt.

Equipment

For measurements of fluxes (oxygen and nutrients) diffusion chambers were constructed. Modifications to chambers used by others (Hall and Holby, 1986; Nixon et al., 1976; Pamatmat and Fenton, 1968) were the relatively large size, about 160 liters, and the gas sampling device. The top of the chambers were conical, at the apex of which was a graduated cylinder making readings of the position of the gas-water interface possible. In one chamber an oxygen electrode was positioned for continuous monitoring of the oxygen concentration.

For measurements of water characteristics, an oxygen electrode and a salinometer together with a current meter were used.

Cores for collecting sediment were constructed of plexiglass and supplied with rubber corks.

To collect gas for composition analysis a gas sampling device was constructed consisting of a plexiglass pyramid of 1 m^2 with a 300ml collecting flask on top.

Field Sampling

From February 1987 to April 1988 a sampling program was exercised four times.

Oxygen consumption of the sediment was measured together with the release/uptake of nutrients (ammonia, nitrate, phosphate, silicate) by means of two diffusion chambers. Samples were collected by divers four times during the 4-5 hours the measurements lasted, and the amount of gas released from the sediment was measured during the same period.

To follow the distribution of the waste through the food chain, samples were taken for measurement of the ratio of the stable isotopes of carbon and nitrogen in food, farmed fish, fecal pellets, sediment, bottom living animals and wild fish.

Water characteristics such as temperature, salinity, oxygen and current were measured throughout the water column (results not presented here).

On six occasions, the amount of resistant bacteria and the concentration of antibiotics in the sediment was measured.

Sediment cores were collected by divers and later the top 5 cm were analysed for ash- free dry weight, C-N-P ratio, and a number of samples were investigated for antibiotics, bacterial quantification and resistance.

Measurements of the gas composition close to the sediment surface and up through the water column were made.

Lab Sampling

Five aquaria (130 liters), supplied with a 5 cm thick layer of shell sand, were topped with a layer of organic waste from a fish farm. A 3 mm layer ground medicated dry pellets containing oxytetracycline was placed in four aquaria, in two of which the pellets were covered with an additional 4 cm layer of organic waste. Samples of sediment were collected by means of small plexiglass cores.

Chemical analysis

Oxygen was analysed by the Winkler method and ammonia was analysed according to Koroleff (Grasshoff et al., 1976) with brief modifications.

Nitrate, phosphate and silicate were automatically analysed according to Chem Lab Instruments Continuous Flow Analysis.

Organic carbon and nitrogen was measured with a Carlo Erba CHN analyser and total phosphorus was analysed according to Koroleff (op. cit.).

Ash free dry weight was determined by combusting the sediment for 24 hours at 450^{0}C.

Stable isotopes were measured on a Finnigan Mat mass spectrometer (with modifications) at the National Lab for Light Stable Isotopes, Univ. of Bergen.

Oxytetracycline was determined according to Samuelsen (1988, in prep) and gas samples were measured on a Hewlett Packard 5992 GLC/MS system.

Bacterial Analysis

Plate counts of bacteria were determined by spreading appropriate dilutions of sediment samples on standard media (Marine Agar, MA, and Tryptone Soya Citrate Agar, TSC), with and without antibiotics (see Torsvik et al., 1988).

Total counts were determined by epiflourescence microscopy of samples stained with DAPI (4', 6-diamidino-2-phenylindole) using the technique of Porter and Feig (1980). The bacteria were counted in a Leitz Orthoplane microscope with a Ploemopak A filter.

Numbers of sulphate-reducing bacteria (SRB) were determined by the most probable number (MPN) technique on SRB medium with lactate as carbon source (Psennig et al, 1981). 0.5 grams of sediment were added to 50 ml medium which had been flushed with N_2. Tenfold dilutions were made and the cultures were incubated at 30^{0}C for 30 days.

Phenotypical diversity of oxytetracycline-resistant isolates were determined using the following tests: colony morphology, colony colour, cell morphology, motility, Gram-reaction by the KOH test (Buck, 1982) oxidative or fermentative metabolism, and presence of the enzymes oxidase and catalase.

RESULTS

Table 1 shows a list of measurements from the various fish farms and the control station. The farms are ranked according to accumulation of organic matter at the sediment surface under the farm. Farm E was abandoned a year before the investiagtion started in February, 1987, and farm F had become the new location, but halfway through the investiagtion the farm was moved back to location E, leaving location F as the abandoned site. Fish farm G was moved in the autumn of 1987 and was not followed further.

Samples for bacterial counts were taken at farm E when the site was abandoned and the sediment had not received antibiotics for 18 months. The samples from farm D were taken during an outbreak of coldwater vibriosis (Vibrio salmonicida) which was treated with oxytetracycline.

The number of aerobic/facultative anaerobic bacteria decreased after medication with oxytetracycline (Table 2). In one sediment (B) the percent of oxytetracycline-resistant bacteria was nearly the same before and after the treatment. Samples from untreated sediment from farm E were not available, but twelve days after treatment the number of resistant bacteria in this sediment was extremely high. After 74 days, the resistance level decreased and was nearly the same in the two sediments. Farm E was also treated with furazolidone and the percent resistant bacteria was 7% at day 74.

In the aquaria in the laboratory, the persistance of oxytetracycline in sediments (Fig. 1, a and b) show a rapid decrease in concentration within the first few days. The oxytetracycline persisted longer in the sediments where it was covered, simulating a heavy precipitation of organic matter after the cessation of medication.

From media with oxytetracycline 50 bacteria strains were isolated. The strains were screened for resistance to 5 antibiotics, and those

having multiple resistance were screened for plasmids (Torsvik et al., op. cit.). The 45 strains isolated having multiple resistance were charcterised phenotypically and grouped by cluster analysis. Highest phenotypic variation was found among strains isolated from the control sediment.

Table 4 shows the gas composition just above the sediment surface at five fish farms. The sediment was manually disturbed leading to a massive escape of gas, which may have altered the composition from that of spontaneous bubbling. However, there seemed to be quite a good agreement between farms, especially with regard to the hydrogen sulphide contribution.

DISCUSSION

The amount of organic waste accumulating under the farms will depend on factors like feeding regime, stock density, current and depth under the cages. In this investigation, there is a gradient in the accumulation of waste although the current is weak on all sites but one. However, data on farm management has not yet been treated and so results are still inconclusive.

Macrofauna seems to disappear when the accumulated layer exceeds 10 cm (Table 1), possibly because of both the low consequent oxygen concentrations, the presence of hydrogen sulphide and the lack of suitable substrate (the accumulated layer is extremely flocculent).

The ash-free dry weight of the sediment increases with increasing accumulation up to 10 cm, and the C/N ratio of the control location compared to the farms reveal that the latter are nitrogen enriched. The high degradability of the waste seems to correspond with the bacterial numbers, both of aerobic/facultative anaerobic bacteria and the sulphate-reducing bacteria. Both bacterial groups are considerably more abundant in the farm sediments.

The bacterial activity is again reflected in the oxygen consumption (Kupka Hansen, unpubl. data) where the uptake by the control sediment is negligible, but when organic waste is accumulated the consumption

increases up to 28.5 mmol $O_2/m^2/hr$ on the most loaded sites in the autumn. Seasonal variations are considerable but the oxygen consumption rates measured in this investigation are often high compared to most other investigations, even from fish farms (Hall and Holby, 1985; Avnimelech, 1984; Blackburn et al., 1988).

The sediment probably becomes anoxic immediately below the surface in farms D and E and all bacteria may be subject to anoxia, which seems to agree well with the observation that all the aerobes tested were facultative anaerobes as well.

Maximum bottom temperature was $12^{o}C$ and occurred in the autumn which coincided with spontaneous gas ebullition and, to some extent, with the highest oxygen consumption rates.

Ammonia release from the sediment (Kupka Hansen, unpubl. data) ranged between 0.05 - 5.5 mmol $NH_4/m^2/hr$ and there seemed to be a correlation between high flux rates and high accumulation of waste. Again, autumn brought the highest fluxes.

The results from the aquaria show a rather rapid decrease of oxytetracycline in the sediment right after medication, but the last 50 ppm seem to disappear very slowly (Fig. 1). If there is heavy sedimentation after medication has stopped the initial decrease is a little slower and the persistant amount is considerably higher (Fig. 2).

The sediment from fish farm B had a relatively high level of oxytetracycline-resistant bacteria and the medication did not affect the level significantly (Table 2). The plate counts indicate that part of the bacterial flora was inhibited by the treatment. The extremely high level of oxytetracycline-resistant bacteria in fish farm E may reflect an adaption due to the repeated treatment with this antibioticum. Between days 39-74 the farm was treated anew with both furazolidone and oxytetracycline, as can be seen from the increase in the concentration of the latter.

In the aquaria, sediments supplied with oxytetracycline at the surface had the lowest percent resistance. The control sediment had an

unexpectedly high level of resistance and analysis revealed traces of oxytetracycline in this sediment. The frequency of resistance decreased in the control and the surface-treated aquaria during the experiment but the level was still significant after four months. The bacterial population here has therefore maintained resistance for at least 10 months since treatment had occurred at least 6 months before the sediment was collected.

The difference in phenotypic diversity of the isolates in treated and untreated sediment may be due to inhibition of some of the bacteria when adding high doses of oxytetracycline. The results indicate that even if there are only traces of this antibioticum in the sediment frequency of resistant bacteria and phenotypic diversity may reveal previous exposure to the drug.

During autumn, spontaneous ebullition of gas (Table 4) occurs in the sediment from most of the investigated farms, and although hydrogen sulphide only accounts for about 1.7% this is equivalent to a concentration of about 17,000 ppm. The number of sulphate-reducing bacteria in fish farm sediment is quite high compared to the control and sulphate reduction is most likely a major pathway for decomposition of the accumulated waste material.

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TABLE 1. Overview of samples and sites.

	control station	farm A	farm B	farm C	farm D	farm E	farm F	farm G
FEED TYPE	-	dry	moist	wet/moist	dry	dry/moist	dry/moist	dry
CURRENT (cm/s)	2-8	>20	1-14	2-7	-	1-8	1-8	1-5
DEPTH (m)	10	15	12	15	7	9	11	20
THICKNESS OF ACCUMULATED WASTE (cm)	0	1	4	5	10	20	35	30
ASH FREE DRY WEIGHT (%)	5	6	14	22	67	43	50	43
C/N RATIO	93	-	-	-	14	10	-	-
MACROFAUNA	+	+	+	+	÷	÷	÷	÷
TOTAL BACTERIA	-	-	-	-	1.4×10^9	6.3×10^9	-	-
AEROBIC/FACULTATIVE ANAEROBIC BACTERIA	4.3×10^5	-	-	-	1.9×10^7	1.4×10^7	-	-
SULPHATE REDUCING BACTERIA	10^2	-	-	-	10^6	10^4	-	-

Table 2. Oxytetracycline concentrations, numbers of bacteria and percent resistant bacteria after medication. Plate counts are bacteria per gram wet sediment.

Site	Days after medication	Oxytetracycline concentration ppm	Plate counts	% resistant bacteria
B	before medication	-	3.3×10^7	38
	12	-	1.7×10^6	28
	74	-	2.7×10^5	30
E	12	281	2.3×10^6	68
	39	147	-	-
	74	200	3.0×10^5	29

Table 3. Percent oxytetracycline-resistant bacteria in the mesocosm sediment. Aquaria 1 and 2 are parallels where the antibioticum was placed on top of the sediment. Aquaria 3 and 4 are parallels where the oxytetracycline was covered with a layer of organic waste. Aquarium 5 is a control. Plate counts are bacteria per gram wet sediment.

		AQUARIA		
	DAY	1 and 2	3 and 4	5
PLATE COUNTS	8	1.7×10^7	1.6×10^6	2.4×10^7
	42	1.9×10^7	4.9×10^7	2.0×10^7
	107	1.6×10^7	2.2×10^7	1.8×10^7
PERCENT RESISTANT BACTERIA	8	6.1	30.6	19.3
	42	5.9	20.0	12.5
	107	9.0	12.0	9.3

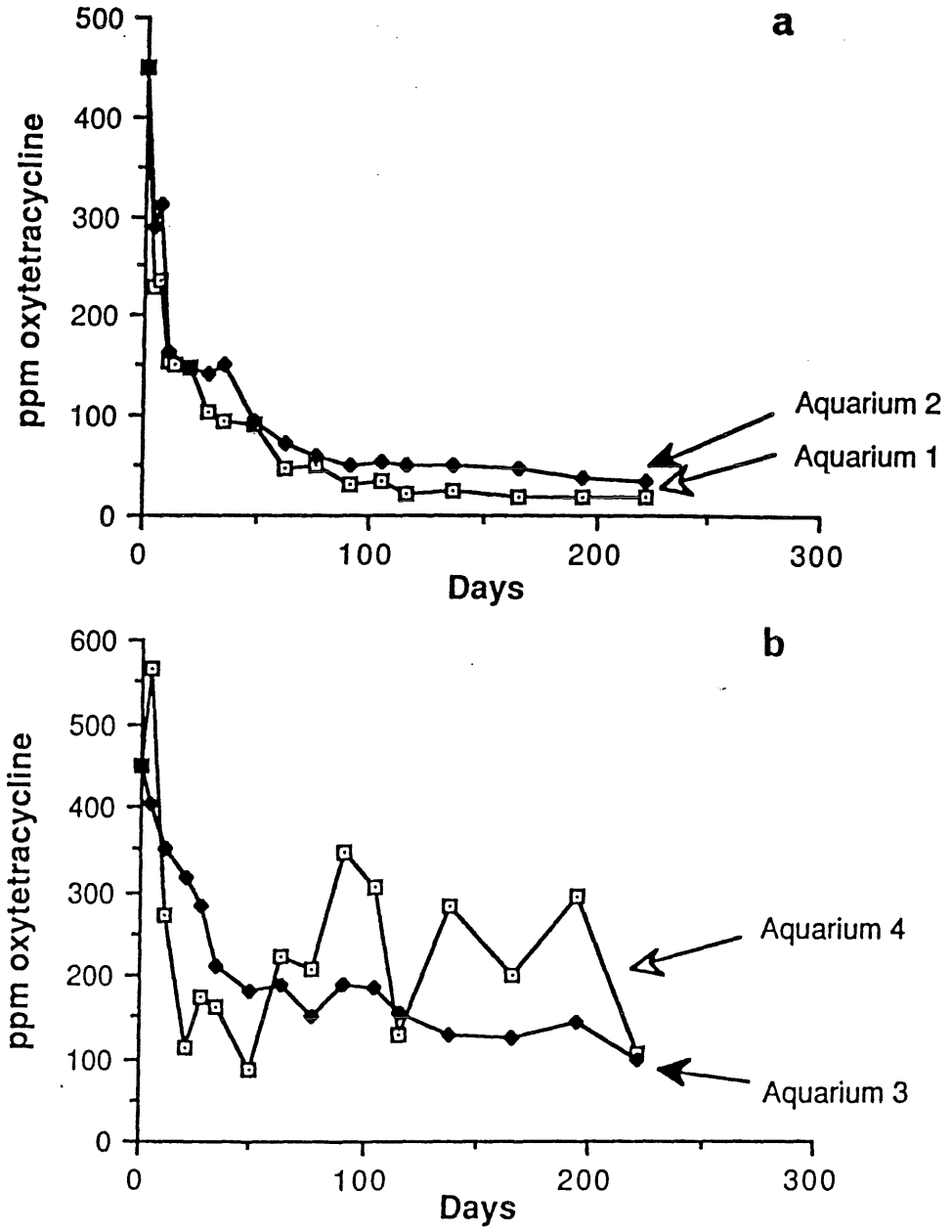

Fig. 1 (a and b) Persistance of oxytetracycline in the mesocosm sediment. Aquaria 1 and 2 (Fig. 1a) are parallels where the antibioticum was placed on top of the sediment. Aquaria 3 and 4 (Fig. 1b) are parallels where the oxytetracycline was covered with a layer of organic waste.

Table 4. Composition of gas sampled just above the sediment surface at five fish farms. The values are an average of four samples.

Farm nr.	% CH_4	% CO_2	% H_2S	sediment thickness
1[a]	88.1	10.0	1.9	24 cm
2[a]	89.0	9.2	1.8	25 cm
3[a]	84.7	13.7	1.6	12 cm
4[a]	78.2	20.2	1.6	14 cm
5*	68.3	30.0	1.7	-

- no measurement
* sampled in October 1985.
a sampled in February 1986.