Vol. 9: 67–72, 1990 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published August 16

Reduction in the antibacterial effect of oxytetracycline in sea water by complex formation with magnesium and calcium

Bjørn T. Lunestad, Jostein Goksøyr

Department of Microbiology and Plant Physiology, University of Bergen, Jahnebakken 5, N-5007 Bergen, Norway

ABSTRACT: Oxytetracycline (OT) is used extensively in the treatment of bacterial diseases in marine fish. The standard dose recommended for treatment of fish is 5 to 10 times higher than doses commonly used in medical practice, indicating that OT is poorly absorbed. This is especially true for fish held in sea water, where the intestinal uptake is substantially reduced as compared to fish in fresh water. OT forms complexes with magnesium and calcium. We have determined the complex constants and calculated that when therapeutic concentrations of OT are present in sea water, only about 5 % of the OT exists in the free form. Of the bound OT, the 1:1 OT-magnesium complex predominates. The complex formation in sea water results in a strong reduction in the antibacterial effect of OT. This has been demonstrated in vitro. The poor intestinal uptake and reduced antibacterial effect of complex-bound OT is caused by an alteration of the molecular charge that diminishes its ability to cross lipid-rich biological membranes. A major portion of the OT administered to farmed salmonids inevitably ends up in the environment, especially in the sediments under aquaculture facilities. No mechanism is known for biodegradation of OT and thus it can remain in the sediments long enough to affect the indigenous bacterial flora and induce resistance. For these reasons the use of OT in marine environments should be questioned.

INTRODUCTION

In the treatment of bacterial diseases in marine fish, oxytetracycline (OT) has often been the agent of choice. Even though a reduction in the use of OT has been observed in recent years, considerable amounts are still prescribed. In 1988 the amount of OT used in Norwegian aquaculture was 18 200 kg, measured as the active component. The corresponding value for 1989 was 5000 kg.

Tetracyclines have a number of potential metal binding sites (Gulbis & Everett 1976, Day et al. 1978, Lambs et al. 1984). The ability of tetracyclines to form complexes with di- and trivalent cations has long been known (Albert 1953, Albert & Rees 1956, Clive 1968). This ability is most strongly pronounced for hydrophilic tetracyclines (mainly tetracycline and oxytetracycline). Lipophilic tetracycline derivatives, such as doxycycline and minocycline do not exhibit the same ability to form complexes (Goodman & Gilman 1985). The presence of di- or trivalent cations has also been shown to mediate the binding of tetracyclines to macromolecules (Kohn 1961).

Sea water with a salinity of 35 ‰ contains 54 mM Mg^{2+} and 10 mM Ca^{2+} (Potts & Parry 1964). The concentration of other di- or trivalent cations is negligible in sea water. In this study, we show, using in vitro tests, how complex-formation with Mg^{2+} and Ca^{2+} affects the antibacterial activity of OT.

MATERIAL AND METHODS

The complex constants for OT-hydrochloride (Sigma Chemical Company, USA) with magnesium and calcium were determined spectrophotometrically (Shimadzu UV-240 spectrophotometer). OT was added to buffered solutions containing magnesium or calcium at concentrations similar to those found in sea water. The OT was added to yield concentrations commonly used for minimum inhibitory concentration (MIC) testing (2 to 100 µM; Mwt 496.9). The large excess of bivalent cation used ensured that the complex formed was mainly 1:1. Because the ability to form complexes is pH dependent, all measurements were done in a 12.5 mM borate/HCl buffer solution, pH 8 (Dawson et al.

0177-5103/90/0009/0067/$ 03.00

1969). The ionic strength was adjusted to that of sea water using NaCl. Investigations carried out by White & Pearce (1982) on chlorotetracycline (aureomycine) revealed a maximum complex binding at pH 8. At this pH, 1:1 complexes are formed.

A freshly prepared stock solution of 1 mM OT in borate/HCl buffer was diluted to appropriate concentrations with the same buffer. Absorption spectra were recorded to give the spectrum of OT alone. Then $CaCl_2$ or $MgSO_4$ was added until no further change in the absorption spectrum was observed. Under these conditions, OT exists predominantly as a 1:1 complex with the added cation. When the absorption characteristics of free and complex-bound OT had been determined, their specific contribution to the total absorption at a given wavelength could be obtained. Thus the complex constant could be calculated from the concentrations of free OT, complex-bound OT and the cation in accordance with the equation:

$$K = \frac{C\ MeOT}{C\ Me \cdot C\ OT}$$

C MeOT is the concentration of metal-OT complex, C Me the concentration of free metal, C OT the concentration of free OT and K the complex constant. The initially added OT equals C MeOT + C OT, and the added metal equals C MeOT + C Me.

Bacterial sensitivity to OT was determined using the MIC method and the disc-diffusion test. In all tests the medium used was Tryptone Soya Agar (TSA) which is recommended by the producer, Oxoid, for sensitivity testing. The medium was prepared with Trypton Soya Broth, Oxoid (20 g l^{-1}) and Special Agar Noble, Difco (15 g l^{-1}) the pH being adjusted to 8 with borate buffer. The agar was supplemented with either 25‰ NaCl, 70 % seawater, 10 mM Ca^{2+} (as $CaCl_2$), 10 mM Mg^{2+} or 54 mM Mg^{2+} (as $MgCl_2$). In all cases the ionic strength was adjusted to a level equal to 25‰ NaCl. For MIC evaluation the strains were plated on TSA containing doubling concentrations of OT in the range 1 to 256 µM (0.5 to 128 µg ml^{-1}). For disc-diffusion tests, a thin lawn of bacteria was spread over the agar surface with a cotton swab as recommended by the disc producer (Oxoid). Discs containing 30 µg OT were placed on the lawn of bacteria before incubation. Inhibition zones were measured as the distance from the edge of the disc to the border of uninhibited bacterial growth.

The antibacterial effect of OT was investigated on 3 well described bacterial strains *Escherichia coli* JM-103, *E. coli* B6 and *Yersinia ruckeri* RS 11^T (ATCC 29473). *Y. ruckeri* RS 11^T has been shown to grow on media containing 6 % NaCl (G. H. Hansen, this department, pers. comm.) *Y. ruckeri* has been shown able to infect fish held in sea water (Sparboe et al. 1986).

When medicating diseased fish, the therapeutic agent is usually coated on food pellets. A major part of the OT given in this way ends up in the environment, either directly as a result of wasteful over-feeding or indirectly via the faeces. Particle-bound OT will, provided the water current is moderate, settle in the vicinity of the aquaculture facility. The sediment will thus receive considerable amounts of OT. To examine the antibacterial effect of complex-bound OT on sediment bacteria we included 5 randomly chosen strains isolated from sediments under marine aquaculture facilities in our investigations. These bacteria were Gram-negative, motile, oxidase-positive, rods. For the antibiotic sensitivity tests, the 2 strains of *Escherichia coli*, were incubated for 24 h at 30 °C, *Yersinia ruckeri* was incubated for 48 h at 22 °C, and the 5 strains of sediment bacteria were incubated for 72 h at 15 °C. The results were read following these incubation times.

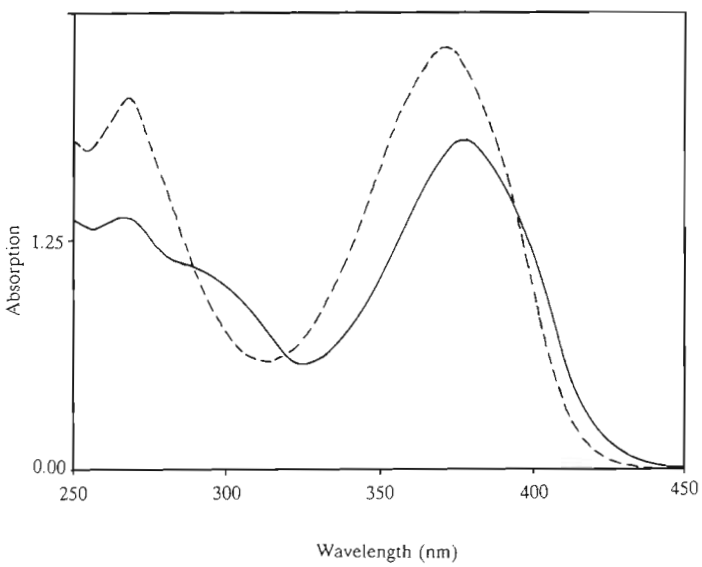

Fig. 1. Absorption spectra of 100 µM OT in borate/HCl buffer solutions (pH 8) of 54 mM Mg^{2+} (– – –) or 10 mM Ca^{2+} (——). The ionic strength was adjusted to the sea water level with NaCl

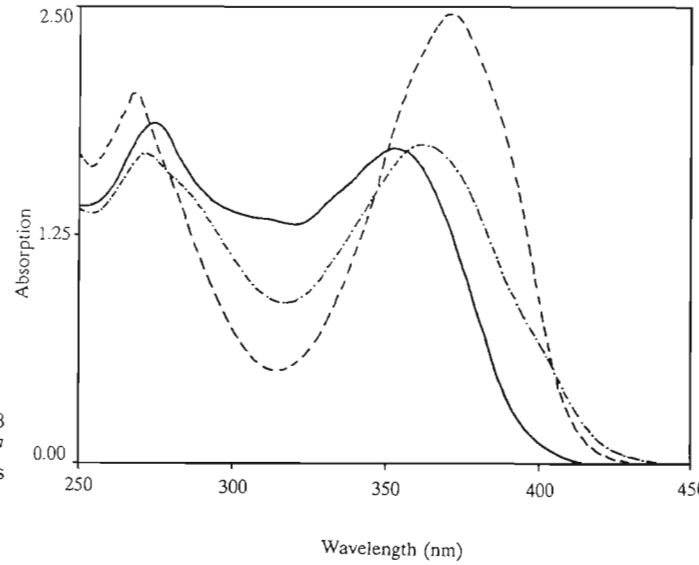

Fig. 2. Absorption spectra of 100 µM OT in sea water pH 8 (– – –), borate/HCl buffer pH 8 (– · –) and distilled water pH 7 (——). The ionic strength in the buffer and distilled water was adjusted to sea water level with NaCl

In a separate disc-diffusion experiment, we investigated how the amount of sea water in the TSA-medium affected the size of the inhibition zones.

RESULTS

Figs. 1 and 2 show the absorption spectra of 100 µM OT dissolved in buffered or unbuffered distilled water, in borate/HCl buffer solution with 54 mM Mg^{2+} or 10 mM Ca^{2+} and in sea water Both the pH of the solution and the presence of divalent cations altered the spectra. The spectrum of OT in sea water was very similar to that of OT in a buffered solution with 54 mM magnesium.

The complex constant for a 1:1 complex between Mg^{2+} or Ca^{2+} and OT at pH 8 was determined using the absorption values at 390 nm, where the highest difference in absorption between free and complex-bound OT was observed. The absorption of OT complexes was found using a cation concentration of 0.1 M. There was no increase in the absorption when increasing the concentration of the cation above 0.1 M, indicating that the complex-binding was close to 100 %. Molar absorption values were found to be 10 000 cm^{-1} for free OT, 13 200 cm^{-1} for MgOT, and 16 500 cm^{-1} for CaOT. The values used for calculating the complex constants are listed in Table 1. The constants were found to be 2.9×10^2 M^{-1} for MgOT and 1.9×10^2 M^{-1} for CaOT, indicating that Mg^{2+} is the most avid ligand. The complex constants given above are the mean of the values from Table 1.

The addition of magnesium or calcium to the medium led to a strong reduction in the antibacterial activity of

Table 1. Determination of complex constants for MgOT and CaOT, and calculation of free OT in 35 ‰ sea water when present in therapeutic concentrations (0.5 to 100 µg ml^{-1}). The complex constants have been determined in 35 ‰ NaCl (to give the same ionic strength as in sea water), buffered to pH 8.0 with 12.5 mM borate buffer. Molar absorption at 390 nm was 10 000 cm^{-1} for OT, 16 500 cm^{-1} for CaOT and 13 200 cm^{-1} for MgOT

Cation conc.	OT conc.	A_{390}	K	Free OT
Mg^{2+}				
10^{-2} M	5×10^{-5} (M)	0.62	300	1.25×10^{-5} (M)
10^{-2} M	10×10^{-5} (M)	1.23	285	2.50×10^{-5} (M)
Ca^{2+}				
10^{-2} M	5×10^{-5} (M)	0.71	185	1.75×10^{-5} (M)
10^{-2} M	10×10^{-5} (M)	1.43	195	3.38×10^{-5} (M)
Sea water				
(54 mM Mg^{2+},	0.5 (µg ml^{-1})		Calculated	0.027 (µg ml^{-1})
10 mM Ca^{2+})	5.0 (µg ml^{-1})		Calculated	0.27 (µg ml^{-1})
	50 (µg ml^{-1})		Calculated	2.7 (µg ml^{-1})
	100 (µg ml^{-1})		Calculated	5.3 (µg ml^{-1})

Table 2. The separate contributions of Mg^{2+} and Ca^{2+} in reducing the antibacterial effect of OT expressed as inhibition zone sizes on TSA. Inhibition zones obtained when using discs containing 30 µg OT are given as the distance in mm from the edge of the disc to the border of uninhibited bacterial growth. V.257, V.369, E.112, E.177 and E.189 are Gram-negative, oxidase-positive, motile rods isolated from a marine aquaculture sediment. They are included as representatives for bacteria in the environment

Strain	25‰ NaCl	10 mM Ca^{2+}	10 mM Mg^{2+}	54 mM Mg^{2+}
Escherichia coli B6	11.0	9.0	7.0	3.0
E. coli JM-103	8.5	7.0	5.0	0.0
Yersinia ruckeri RS-11^T	10.5	8.0	5.5	0.0
V.257	2.0	1.5	1.0	0.0
V.369	13.0	6.0	5.0	3.0
E.112	12.0	5.5	4.0	1.5
E.177	15.5	5.0	3.5	0.0
E.189	13.0	9.5	8.5	5.5

OT as shown in Table 2. In equimolar concentrations, Mg^{2+} exhibited a greater ability than Ca^{2+} at reducing the effect of OT. This was in agreement with the complex constants previously determined.

In Figs. 3 and 4 the size of the inhibition zones obtained on TSA with antibiotic discs were plotted against the amount of sea water added to the medium. For all strains, the sizes of the inhibition zones were reduced by more than 55% when tested on TSA prepared with 100% sea water as compared to TSA prepared with 35‰ NaCl in pH-adjusted distilled water (pH 8).

MIC tests were carried out with 70% sea water or with varying concentrations of Mg^{2+} or Ca^{2+}. The results are listed in Table 3. In all cases, addition of Mg^{2+}/Ca^{2+} as sea water or as chemicals led to a profound increase in the ability of the bacteria to tolerate OT. The effect of Mg^{2+} was greater than the effect of Ca^{2+} as reported above.

To eliminate the possibility that the reduction in antibacterial effect was due to a reduced diffusion rate of OT complexes in the agar, experiments were also carried out in liquid media with agitation. The results confirmed the findings obtained using agar media (data not shown).

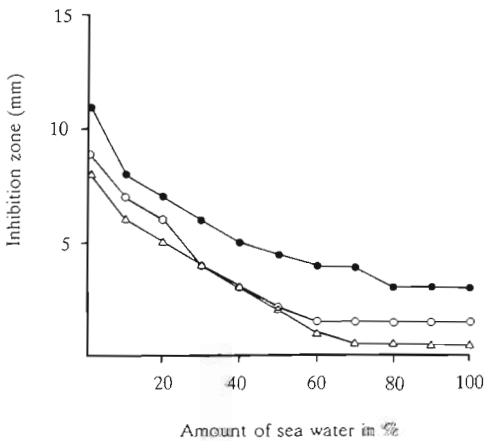

Fig. 3. Inhibition zones (mm) for *Escherichia coli* JM-103 (▲), *E. coli* B6 (●) and *Yersinia ruckeri* RS 11^T (ATCC 29473) (○) tested by agar diffusion using discs containing 30 µg oxytetracycline. Inhibition zones where plotted against the amount of sea water added to Tryptone Soya Agar. Salinity was adjusted to 35‰ with NaCl and the pH to 8. Inhibition zones were measured as the distance from the edge of the disc to the border of uninhibited bacterial growth

DISCUSSION

Because the effect of some antibacterial agents is influenced by cations in sea water, media used for testing antibiotic resistance of marine bacteria should

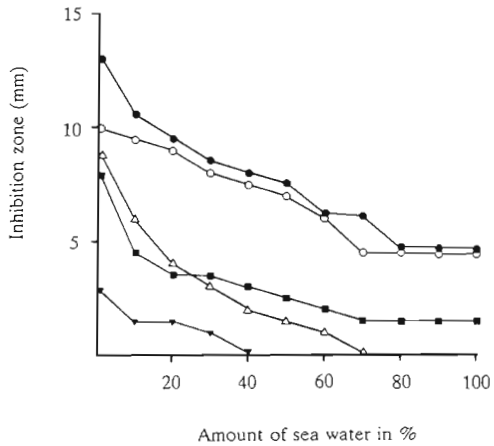

Fig. 4. Inhibition zones (mm) for 5 randomly chosen strains of sediment bacteria isolated from under aquaculture facilities. The strains were not identified but were all Gram-negative, oxidase-positive, motile rods. Strains V.257 (▼), V.369 (▽), E.112 (■), E.177 (△) and E.189 (●) were tested on Tryptone Soya Agar (TSA) using discs containing 30 µg oxytetracycline. The inhibition zones are plotted against the amount of sea water added to the agar. The salinity was adjusted to 35‰ with NaCl and the pH to 8. The inhibition zone were measured as the distance in mm from the edge of the disc to the border of uninhibited bacterial growth

Table 3. Minimum inhibitory concentration (MIC) on TSA media containing various concentrations of seawater, Mg^{2+} or Ca^{2+} The MIC values are given as µg OT ml^{-1}

Strain	25 ‰ NaCl	70 % sea water	10 mM Ca^{2+}	10 mM Mg^{2+}	54 mM Mg^{2+}
Escherichia coli B6	0.5	4	1	4	32
E. coli JM-103	2	16	4	16	64
Yersinia ruckeri RS-11^T	1	32	1	4	32
V.257	32	>128	32	>64	>64
V.369	0.5	4	0.5	4	16
E.112	0.5	2	4	16	>64
E.177	4	16	2	16	8
E.189	0.5	2	0.5	1	

be prepared with natural or synthetic sea water containing Mg^{2+} and Ca^{2+}. This will give a more correct picture of the actual antibacterial activity. We therefore recommend that an addition of 70 % natural sea water or an equivalent amount of synthetic sea water should be set as a standard. The amount of Mg^{2+} and Ca^{2+} in commercial synthetic sea waters is approximately the same as found in natural sea water. Thus, 'Rila Marine' (Rila Products, Teaneck, NJ USA) contains 51.8 mM Mg^{2+} and 12.2 mM Ca^{2+}; 'Instant Ocean' (Aqarium Systems, Ohio, USA) contains 54.6 mM Mg^{2+} and 9.7 mM Ca^{2+}; and 'Ultra Marine' (Waterlife Research Industries Ltd., UK) contains 53.3 mM Mg^{2+} and 10.0 mM Ca^{2+} when diluted with distilled water as recommended by the producers.

A considerable underestimation of the level of OT resistance may occur when media prepared without sea water are used. In sea water, a calculated 95 % of the OT, added at concentrations of from 0.5 to 100 µg ml^{-1}, is bound by the magnesium and calcium ions present in the water (Table 1). This binding is associated with a substantial loss in the antibacterial activity of OT, making it necessary to use considerably more OT to obtain the same effect as that obtained in the absence of these ions. This effect is clearly shown in Table 3 where the presence of 70 % sea water in the MIC test medium increased the MIC values for the various organisms tested by an average of almost 10-fold (range 4 to 32-fold) over those obtained on the medium containing NaCl in place of sea water. The 10-fold decrease in the efficiency of OT detected with sea water using the MIC assay does not quite equal the 20-fold decrease predicted by the spectral data in Table 1, perhaps because of the imprecision built into the MIC assay method. Notwithstanding this, it is clear that the sensitivity of micro-organisms to OT in sea water will have to be determined on media containing calcium and magnesium ions if realistic estimates of their OT sensitivity are to be obtained.

Clinical reports have shown a clear reduction in gastrointestinal uptake of OT when administered orally with simultaneous intake of food containing di- or trivalent cations (Neuvonen 1976, Cunha et al. 1982). When seawater fish are medicated, OT will unavoidably come in contact with Mg^{2+} and Ca^{2+}. This occurs when the OT-coated food pellet is in the sea water or in the gastrointestinal tract. Marine teleosts are hypoosmotic and must drink sea water to compensate for loss of water. Rainbow trout *Salmo gairdneri* kept in sea water have been reported to drink 129 ml kg^{-1} d^{-1}, while rainbow trout kept in fresh water drink little or not at all (Usher et al. 1988). Drinking rate was independent of gut food content but was significantly higher in feeding fish (Usher et al. 1988). Thus, under sea water conditions the gut fluid can be regarded as slightly modified sea water. Because OT in the intestine must be taken up from the liquid phase, the presence of Mg^{2+} and Ca^{2+} will affect the absorption. This has lately been confirmed by Ellingsen et al. (unpubl.). Two groups of rainbow trout from the same cohort where medicated, using a standard OT regime, while kept in fresh or sea water. On the average, the tissue level of OT in the sea water group was approximately 30 % of that found in the fresh water group.

To cross a biological lipid membrane, a drug must be undissociated (i.e. uncharged) and not bound to macromolecules (Goodman & Gilman 1985). The reduced effect of complex-bound OT could be explained by the change in molecular charge when it combines with cations. Jun & Lee (1980) have investigated the uptake of tetracycline in red blood cells. A reduction in the uptake was observed when red blood cells were kept in 0.9 % NaCl containing 2.5×10^{-5} M Ca^{2+}. When using the same concentration of Ca^{2+} and NaCl but adding 4 % serum albumin, the uptake was significantly lower than in solutions containing Ca^{2+} or albumin alone. Thus cations probably mediate the binding of tetracyclines to macromolecules by serving as a connecting bridge (Kohn 1961).

Recent studies on tetracycline distribution in blood plasma have revealed that Mg^{2+} and Ca^{2+} complexes have a reduced ability to diffuse through erythrocyte

membranes (Lambs et al. 1988). This effect was also due to alterations in molecular charge and thus the degree of lipid solubility

Because the major part of the OT administered to diseased fish reaches the environment, attention should be paid to the fate and effects of OT in the environment. Samuelsen et al. (1988) found in mesocosm experiments a rather rapid decrease in the OT concentration in the sediment during the first days after medication, but a residue of about 100 µM, or approximately 10 % of the initial concentration, was observed over a long period (225 d). The lost OT was probably not degraded, but washed out from the sediment.

Bacterial resistance to tetracyclines is commonly observed. Tetracycline resistance determinants are the most widespread determinants among all bacterial species (Levy 1984). Despite the presence of a variety of tetracycline-resistant bacteria, no biodegradation pathways for OT are known. No significant loss of antibacterial activity was found when faecal material from animals treated with OT was stored isolated or in contact with soil over a period of months (Levy 1988). The main degradation mechanism for OT in the environment seems to be photodecomposition (Oka et al. 1989).

In view of the widespread observations of OT resistance, the persistent nature of OT, and the reduced uptake of Mg-OT and Ca-OT complexes in fish, the use of this antibiotic in marine environments should be questioned.

Acknowledgement. This work was supported by funds provided by The Norwegian Research Council for Fisheries Science, project no. V 701.231.

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Responsible Subject Editor: Dr T. Evelyn, Nanaimo, B.C., Canada

Manuscript first received: June 30, 1989
Revised version accepted: May 31, 1990