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Inhibition of Aerobic Growth and Nitrification of Bacteria in Sewage Sludge by Antibacterial Agents

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Abstract. Toxicity of antibacterial agents on environmentally relevant bacteria was investigated using activated sludge. The growth and nitrifying inhibiting effects for activated sludge of benzyl penicillin (penicillin G) (BP), tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), olaquindox (O), streptomycin (ST), tiamulin (TI), tylosin (TYL) sulfadiazine (SDZ), metronidazole (MET), and oxolinic acid (OXA) was investigated. Studies were performed in accordance to the ISO 15522 (1999) and ISO 9509 (1989) test guidelines, respectively. The toxicity (EC_{50} value, mg/L) found with the ISO 15522 was in decreasing EC_{50} values; O (95.7), BP (84.6), TYL (54.7), TI (14.3), TC (2.2), OTC (1.2), ST (0.47), CTC (0.40), and OXA (0.1). No observed effect concentrations (NOECs) (mg/L) of 100 and 60, respectively was found for MET and SDZ. Triplicate tests assessing the effects of the antibacterial agents on the nitrification rate gave indications only as the level of increased or decreased rate. More accurate data for the inhibition of *Nitrosomonas europaea* was found with a suspended culture of the nitrifying bacteria. The toxicity (EC_{50} value, mg/L) found was in decreasing EC_{50} values; TI (23.3), SDZ (17.0), TC (4.0), OTC (1.7), OXA (1.0), CTC (0.64), O (0.03), ST (0.02). For MET and TYL, NOECs (mg/L) of 100 and 50 were found, respectively. The antibacterial agents were also assessed using a pour plate method with both (separately tested) activated sludge bacteria and *N. europeae* showing to be 5 to 10 times more potent to most agents except SDZ, TI, and MET.

By several pathways (e.g., nondegradable residues in the sewage treatment plant (STP), residues in manure and sludge used as fertilizer on fields or from surplus drugs in aquaculture) antibacterial agents may reach the environment (Halling-Sørensen *et al.* 1998; Jørgensen and Halling-Sørensen 2000). More than 25% of the antibacterial load to the environment in Denmark is emitted from the STP with feces and urine from humans treated against infectious diseases (Halling-Sørensen *et al.* 1998). Besides causing development of antibacterial resistance, antibacterial agents or their residues may reduce or alter the capacity of activated sludge in the STP to degrade other organic xenobiotics or to nitrify ammonia due to their bacterial potency. If the activated sludge is used as soil conditioner, antibacterial agents and residues sorbed to the sludge may also affect the soil bacterial community. Gram-negative bacteria, *e.g., Nitrosomonas* sp., run the nitrification process. Only Gram-negative affecting antibacterial agents, such as tetracyclines, aminoglycosides, sulfonamides (all having a broad spectrum of activity), or quinolones (narrow spectrum of activity) are therefore expected to inhibit the nitrification. The potency of antibacterial agents against target pathogenic bacteria (often expressed as the *minimal inhibitory concentration* [MIC value]) (Sayed *et al.* 1998; Ziv 1980) or against environmentally relevant single bacteria species, such as *Vibrio fischeri*, is well known at least for some antibacterial agents (Backhaus *et al.* 1997; Backhaus and Grimme 1999). Backhaus and co-workers did not investigate effects of antibacterial agents to bacterial communities (nontarget bacteria), *e.g.*, sludge, or functions of the sludge (nitrification).

The aim of the present study was to answer this question by assessing the potency of selected antimicrobial agents belonging to different compound classes using standardized short-term tests (duration up to 6 h) (*e.g.*, ISO 15522 [ISO 1999] and ISO 9509 [ISO 1989]). Several problems arise using bacterial communities (mixed bacterial cultures), *e.g.*, sludge, instead of single species to assess the toxicity of antibacterial agents. First, some antibacterial agents act with a high degree of selectivity against specific target bacteria. Only antibacterial agents that posses a broad spectrum of activity will affect the entire sludge bacterial community. Antibacterial agents with a narrow spectrum of activity will, on the other hand, only affect a certain part of the bacterial community. This may result in a shift in the microbial sludge population enabling unaffected species to create dominants and potentially alter the activity of the sludge, *i.e.*, reduce the nitrification capacity. Backhaus *et al.* (1997) already showed that short-term bacterial test (*V. fischeri*) for specific acting chemicals might be less sensitive than tests with longer duration. This was found for tetracyclines and chloramphenicol where the ratio between acute and chronic EC_{50}s were factors of 825 and 1,335, respectively. Second, to prolong the test duration to 3 days or more I furthermore assessed the antibacterial agents with a pour plate

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method with activated sludge bacteria and *Nitrosomonas europeae* (separately tested). Finally, the ratio of antibacterial resistant colonies in the sludge to different antibacterial agents was assessed.

The antibacterial agents assessed (all used in Denmark) in this investigation are: *benzylpenicillin* (penicillin G) (BP), *tetracycline* (TC), *chlortetracycline* (CTC), *oxytetracycline* (OTC), *olaquindox* (O), *streptomycin* (ST), *tiamulin* (TI), *tylosin* (TYL), *sulfadiazine* (SDZ), *metronidazole* (MET), and *oxolinic acid* (OXA). Chemical structures are shown in Figure 1. Table 1 shows chemical name, relevant physicochemical data, and CAS numbers, respectively. Antibacterial agents are often water-soluble (except TI) and often mono- or polyprotic compounds therefore the bioavailability of the compounds are highly pH-dependent (Holten Lützhøft *et al.* 1999). Table 2 shows the spectrum and mode of action for the selected compounds. The compounds represent different groups of antibacterial agents with different mode of action; β-lactams, BP; tetracyclines, OTC, CTC, and TC; macrolids, TYL; aminoglycoside, ST; and quinoxaline 1,4-di-N-oxides, O; quinolones; OXA; sulfonamides; SOZ; imidazole; MET. TI is a pleuromutilin derivative.

Materials and Methods

Chemical Substances

Test compounds (purity % if available) were purchased from the following companies: $K_2Cr_2O_7$ (> 99.8%, Riedel-de Haën, Seelze, Germany); 3.5 dichlorphenol (Sigma Chemical Company, St. Louis, MO) (both used as reference chemical). Phenol and 2,3-dichlorphenol (Sigma) (used as positive controls in the nitrification test). Tiamulin hydrogen fumerate (TI) (lot no D2766, active compound 81.0%, Leo Pharmaceutical Products, Ballerup). Oxolinic acid (> 98%) (OXA) and oxytetracycline hydrochloride (OTC) (lot no. 28555, 95.7%, Unikem, Copenhagen). Tylosin tatrate (TYL) (active compound 89.8%), tetracycline (TC), penicillin G sodium salt (BP), chlortetracycline hydrochloride (CTC), spiramycin (mixture of I, II, and III) (SP), all from Sigma. Streptomycin sulfate (SP, ICN Biochemicals, Aurora, OH), Olaquindox (O) (98.16%, Yick-Vic, Chemicals and Pharmaceuticals, Hong Kong). Chemicals used for growth medium were all of analytical grade and purchased from Merck, Denmark.

As surface active ingredient in the pour plate test polyoxyethylene sorbitan mono oleale (Tween 80) P-4780 from Sigma was used. N-allylthiourea from Merck was used in the nitrification test as specific inhibitor. The following chemicals were all used for analyzing nitrate, nitrite, and ammonium: 4-aminobenzen sulfonamide (28H1005, Sigma), N-(1-naphthyl)-ethylendiamine-dihydrochloride, and NaN_3 both from Merck, Denmark.

Test Solutions

Because of the limited stability in aqueous solution of several of the tested antibacterial agents (*e.g.*, tetracyclines and β-lactams) all compounds were dissolved in purified water (Milli-Q) shortly before testing. Initial pH in all test solutions was between 8.1 and 8.3. The pH was measured with a PHM95 pH/ion METER, Radiometer Denmark, A/S. Concentrations of the antibacterial were not determined by analytical chemical methods; the respective nominal concentration was used as exposure concentration in the calculations of EC_{50} values.

Activated Sludge Bacteria

Tests were performed with activated sludge from the primary aeration tank at a pilot-scale activated sludge sewage treatment plant receiving municipal wastewater (Institute of Environmental Science and Technology, Lyngby, Denmark). Precondition (aeration) of the sludge began within 1 h of collection and took 20 h at room temperature.

Growth Inhibition of Activated Sludge

The growth inhibitory effect of antibacterial agents on sewage microorganisms was performed in accordance with the ISO 15522 (ISO 1999) guideline. Detailed informations about media constituents, preculture and main culture preparation and test performance is explained in ISO 15522 (ISO 1999). All tests were made in accordance with the test protocol. A test consisted of the following samples; six control replicates, six concentration levels of antibacterial agent in duplicate, and five concentration levels of the positive control (3,5-dichlorophenol) in duplicate. The test was ended when exponential growth ceased in the controls (typically after 4–6 h). The bacterial biomass was quantified as turbidity in both control and test vessels spectrophotometrically at OD_{530}nm. Measurements were performed using a Zeiss UV-VIS spectrophotometer model SPECORD S100 (Jena, Germany). The inhibition (I) (%) was calculated as

$$I = [(A - B)/(A - C)] * 100 \quad (1)$$

where A = mean value of measured turbidity at the end of the incubation period in the blank control flasks; B = mean value of measured turbidity at the end of the incubation period in the test flasks; and C = mean value of measured turbidity at the distribution of the main culture when test material is added in the flasks.

Results were reported as the effect concentration to 50% inhibition (EC_{50}). If no effects were observed, a no-effect concentration level (NOEC) was reported as the maximum test concentration in the test design.

Viable Plate Counting (Pour Plate Method) of Activated Sludge Bacteria

Activated sludge bacteria were diluted 100 times in phosphate buffer (17.2 mM K_2HPO_4/7.3 mM KH_2PO_4, pH = 7.2) to obtained a reasonable number of colonizing bacteria on each petri plates (approximately 100 colonies were viable after 3 days in the controls). One milliliter of this bacterial solution was mixed with 7.5 ml agar solution (40–45°C) and 1.5 ml antibacterial agent solution to a final test solution of 10 ml. The agar solution was prepared as prescribed in the DS 2251 (Danish Standard 1983) guideline. The 10-ml final test solution was transferred to a petri plate (9 cm in diameter) and instantly incubated at 21°C ± 1°C for 48 h in a temperate box, allowing the agar to dry. This technique made the bacteria immobilized in agar containing a homogenate concentration of the tested antibacterial agent. Because the bacteria were immobilized in the agar, only well-defined easily counted colonies were developed. Final antibacterial agent concentration in the plates was identical with the concentration applied in the ISO 15522 (ISO 1999) test flasks method (see above). Control plates were produced identically, only instead of antibiotic solution additional buffer solution was added up to 10 ml. Identical numbers of controls, concentration levels of test compound and reference compound (3,5-dichlorophenol) were used as previously described for the growth inhibition test. In addition, a blank reference testing the bacterial contamination of water was applied in all tests.

After 48 h all plates was counted and the inhibition (I) (%) was calculated as

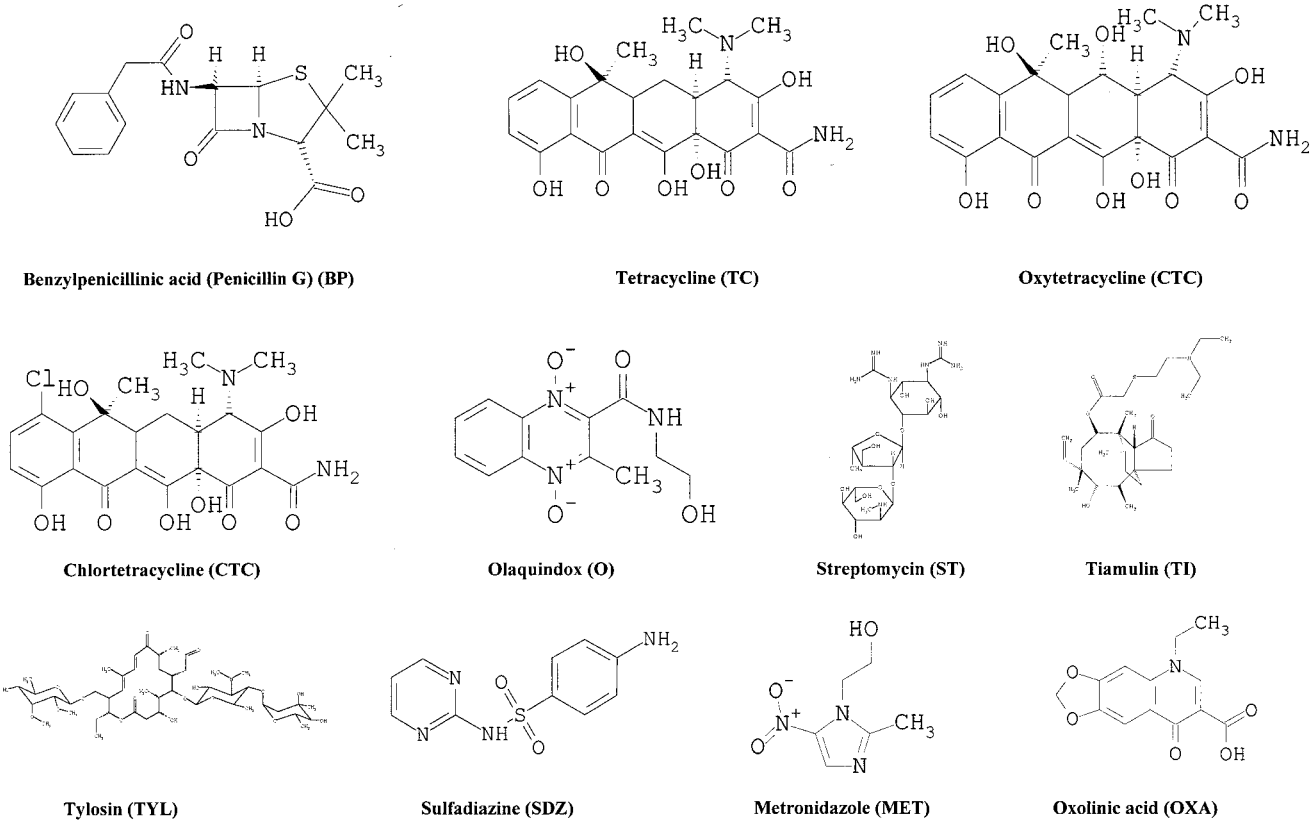

Fig. 1. Chemical structures of the antibacterials tested

$$I = ([D - E]/D) * 100 \quad (2)$$

where D = mean value of colonies counted on the nonexposed agar plates at the end of the incubation period (48 h); E = mean value of colonies counted on the antibiotic treated agar plates at the end of the incubation period (48 h). Data were reported as EC_{50} values (see above).

Bacterial Clumping

Certain bacterial agent may induce bacterial clumping and therefore influence the results using the viable counting method. Bacterial clumping may be reduced by addition to the test solution of nontoxic levels of a nonionic surfactant (*e.g.*, Tween 80) (Sörén *et al.* 1995). Tween 80 was found to be nontoxic to both the suspended test systems and the pour plate method at concentration levels up to 300 mg/L (results not shown). A level of 50 mg/L Tween 80 was therefore used in both the ISO 15522 (ISO 1999) and pour plate method in a comparing experiment. Experiments with OTC, TYL, and O were performed with the same activated sludge sample with and without addition of Tween 80. Tests were otherwise performed exactly as described previously; growth inhibition of activated sludge and viable plate counting of activated sludge bacteria.

Assessment of the Antibacterial Resistance of the Activated Sludge Bacteria

The activated sludge bacteria was assessed for antibacterial resistance as prescribed in the NCCSL (1997) guideline. For each antibacterial agent a susceptible concentration is prescribed. If the antibacterial agent is not susceptible to a certain concentration (*e.g.*, for tetracyclines the concentration is 8 mg L^{-1}) then the strain is considered resistant (NCCSL 1997). The number of colonies on each plate that were not susceptible to a certain high antibacterial concentration (compound specific concentration level) compared to the number of colonies on the controls; this ratio was expressed as the percentage resistant bacteria.

Nitrification Inhibition of Activated Sludge

The effect of antibacterial agents on the nitrification rate in the activated sludge was assessed with the ISO 9509 (ISO 1989) guideline. Detailed informations about media constituents, preculture, and main cultures preparation and test performance is detailed explained in ISO (1989). All tests were made in accordance with the test protocol. Tests consisted of the following samples: six control replicates, six concentration levels of antibacterial agent in duplicate, and five concentration levels of the positive controls (phenol and 3,5-dichlorophenol) in duplicate. A nitrification rate in the control sludge of more than 2 mg (N g^{-1} h^{-1}) as mandatory in the guideline was obtained in all tests. Ammonium, nitrite, and nitrate were determined spectrophotometrically as prescribed in ISO 7150/1 (ISO 1984a), ISO 6777 (ISO 1984b), and ISO 7890/1 (ISO 1986) protocols, respectively. The level of quantification (LOQ) for the three methods were; NH_4^+: 0.19 mg/L, NO_2^-: 37.7 μg/L, and NO_3^-: 1.58 mg/L, respectively. Measurements were performed using a Zeiss UV-VIS spectrophotometer model SPECORD S100.

Table 1. Physicochemical properties, CAS reg. no. of the antibacterial agents tested

Antibacterial Agents	Chemical Structure	CAS Reg. No.	pK_a	Log K_{ow}
β-lactam antibiotics				
Benzylpenicillin (penicillin G) (BP)	(2S,5R,6R)-3,3-Dimethyl-7-oxo-6-phenylacetamido-4-thia-1-aza-bicyclo[3,2,0]-heptane-2-carboxylic acid	69-57-8	2.1	1.72[a]
			2.71[i]	1.76[b]
				1.83[c]
				1.67 ± 0.20[d]
Tetracyclines				
Oxytetracycline (OTC)	4S-(4α,4aα,5a,5aα,6β,12aα)-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphtacenecarboxamide	79-57-2	3.27; 7.32; 9.11[k]	−1.12[o]
Chlortetracycline (CTC)	7-Chloro-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphtacenecarboxamide	64-72-2	3.30; 7.44; 9.27[k]	–
Tetracycline (TC)	(4S,4aS,5aS,6S,12aS)-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-napthathacene-2-carboxamide	60-54-8	3.30; 7.68; 9.69[k]	−1.25[e]
				−1.44[f]
				−1.05[g]
				−1.19 ± 0.71[d]
				−1.4[p]
Quinoxalin				
Olaquindox (O)	(2-[*N*-(2-hydroxyethyl)-carbamoyl]-3-methylquinoxaline-1,4-dioxide	23696-28-8	—	−2.34 ± 1.10[d]
Aminoglucoside				
Streptomycin (ST)	4-*O*-[2-*O*-(2-Deoxy-2-methylamino-α-L-glucopyranosyl)-5-deoxy-3-*C*-formyl-α-L-lyxofuranosyl]-*NN′* diamidino-D-streptamine	57-92-1	—	−3.20 ± 1.04[d]
Pleuromutilin derivative				
Tiamulin (TI)	11-Hydroxy-6,7,10,12-tetramethyl-1-oxo-10-vinylperhydro-3a-7-pentanoinden-8-yl (2-diethylaminoethylthio)acetate hydrogen fumerate	55297-95-5	—	5.93 ± 0.62[d]
Macrolid				
Tylosin (TYL)	Stereoisomer of 15-[[(6-deoxy-2,6-di-*O*-methyl-β-D-allopyranosyl)-oxy]methyl]-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimetyl-2,10-dioxo-oxacyclo-hexadeca-11,13-diene-7-acetaldehyd	1401-69-0	7.73[h]	2.50 ± 0.84[d]
				1.63[h]
Quinolon				
Oxolonic acid (OXA)	5-ethyl-5,8-dihydro-8-oxo-1.3-dioxolo [4,5-g]-quinoline-7-carboxylic-acid	14698-29-4	6.9[l]	0.68[m]
Sulfonamide				
Sulfadiazin (SDZ)	2-(4-Aminobenzene-sulfonamido)pyridine	68-35-9	2.0; 6.5[n]	0.12[o]
Imidazol				
Metronidazol (MET)	1-(2-hydroxyethyl)-2-methyl-5-nitro-imidazole	443-48-1	2.5[p]	−0.1[p]

[a] Ryrfeldt (1971).
[b] Bird and Marchall (1971).
[c] Bird and Marchall (1967).
[d] ACD (1995) log P (estimated for uncharged molecule).
[e] Colaizzi and Klink (1969), measured at pH = 5.6 (phosphate buffer).
[f] Colaizzi and Klink (1969), measured at pH = 7.4 (phosphate buffer, ion corrected).
[g] Miller *et al.* (1977), measured at pH = 6.6 (phosphate buffer).
[h] McFarland *et al.* (1997), calculated from the shift in pK_a value in the presence of rapidly stirred 1-octanol.
[i] Rapson and Bird (1963).
[j] Parke and Davis (1954).
[k] Stephens *et al.* (1956).
[l] Timmers and Sternglanz (1978).
[m] Takács-Novák *et al.* (1992).
[n] Koizumi *et al.* (1964).
[o] Herbert and Dorsey (1995).
[p] Lund (1994) (ocatanol/pH = 7.4).

Table 2. Spectrum and mode of action of the antibacterial agents tested (Mow and Thøgersen, 1997)

Antibacterial Agents	Gram-Positive	Gram-Negative	Anaerobic Bacteria	Bactericidal Action	Bacteriostatic Action	Mode of Action (Merck 1998)
β-lactam antibiotics						
Benzylpenicillin (penicillin G) (BP)	X	(X)		X		Inhibit the development of bacterial cell wall by interfering with transpeptidase enzymes responsible for the formation of the cross linkage between peptidoglycan strands.
Tetracyclines						
Oxytetracycline (OTC)	X	X			X	Bind reversible to bacterial 30 S ribosomes and inhibit protein synthesis.
Chlortetracycline (CTC)	X	X			X	
Tetracycline (TC)	X	X			X	
Quinoxalin						
Olaquindox (O)		(X)		X		Prevents the DNA synthesis in the bacteria and denaturates performed DNA.
Aminoglucoside						
Streptomycin (ST)		X		X		Inhibits by binding irreversible to receptors on both the 30 S and 50 S subunits of bacterial ribosomes, resulting in mall reading the mRNA code.
Pleuromutilin derivative						
Tiamulin (TI)					X	Prevents the microbial protein syntheses, by binding to the 50S portion of the bacterial ribosome.
Macrolid						
Tylosin (TYL)	X				X	Interfering with protein synthesis by reversibly binding to the 50 S subunit of the ribosome.
Quinolones						
Oxolinic acid (OXA)		X		X		Inhibit the bacterial enzyme DNA-gyrase that is responsible for the supercoiling of DNA so that the DNA can twist in a number of chromosomal domains and seal around an RNA core.
Sulfonamide						
Sulfadiazin (SDZ)	X	X			X	Inhibit the DNA/RNA synthesis by competitively inhibiting an enzymatic step (dihydropterate synthetase) during which paraaminobenzoic acid is incorporated into the synthesis of dihydrofolic acid.
Imidazol						
Metronidazol (MET)			X			Metronidazole is transformed in to active metabolites in anaerobic bacteria inhibiting the nuclein syntheses.

Growth Inhibition of N. europaea

A pure culture of nitrifying bacteria (*N. europaea*) was obtained from the Department of Ecology, Section of Genetics and Microbiology, Royal University and Agricultural University, Copenhagen. The same procedure as described above, growth inhibition of activated sludge bacteria, was used. *N. europaea* was inoculated in the growth media and shacked during the entire test period. The test period was expanded to 10 days because of slower growth rate compared to the heterotrophs in the sludge. Because of the slow growth rate of the

nitrifiers, all equipment was sterilized before use. Exponential growth was obtained in the controls during the entire test period. At the end of the 10 days, the ammonium and nitrite content was measured spectrophotometrically in accordance with ISO 7150/1 (ISO 1984a) and ISO 6777 (ISO 1984b), respectively. The inhibition of nitrite production in the test vessels compared to the controls was used as a measure for calculation.

Viable Plate Counting (Pour Plate Method) of N. europaea

The same test procedure and quantification as described above, viable counting of aerobic sludge bacteria, was used. The pure culture (between 20 and 200 bacteria) of *N. europaea* was inoculated in the plates (pour plate method), and viable colonies were calculated after 5 or 6 days (around 100 colonies on the plate). All equipment was sterilized before use because nitrifying bacteria are easily outcompeted by other fast-growing bacteria. To control this I incubated the nitrifying bacteria on both agar plates with ammonium but without glucose and on plates without ammonium but with glucose. The bacterial colonies grew only on the first type, indicating that no contamination was present. After 5 or 6 days (depending of the number of colonies on the plates) the number of viable colonies in the controls (six plates) and tests plates were compared (three replicate plates).

Statistical Treatment of Data

EC_{50} (mg/L) in all tests was determined by weighted nonlinear regression analysis directly on the data using the Weibull equation to describe the concentration response relationship. A computer program developed by Andersen *et al.* (1998) was used to give the EC values and corresponding 95% confidence intervals. The paired experiments with and without addition of the nonionic surfactant were tested statistically by assessing if the 95% confident limit for the two fitting parameters in the Weibull equation overlapped. If the 95% confident limit overlapped the two dose responds curves were considered not statistically different.

Results

Inhibition of Activated Sludge Bacteria

Table 3 shows EC_{50} values (mg/L) for the different antibacterial agents. Results for activated sludge bacteria shows that the antibacterial agents with a broad spectrum of activity (OTC, CTC, TC, ST, TI, OXA, SDZ) with the exception of SDZ generally were more potent than the ones with a narrow spectrum of activity (BP, O, TYL, and MET). The toxicity (EC_{50} value, mg/L) found with the ISO 15522 (ISO 1999) was in decreasing EC_{50} values: O (95.7), BP (84.6), TYL (54.7), TI (14.3), TC (2.2), OTC (1.2), ST (0.47), CTC (0.40), and OXA (0.1). For SDZ and MET no effects were discovered in the tested concentration range why NOECs (the highest tested concentration level) were quantified as 60 mg/L and 100 mg/L, respectively. EC_{50} (mg/L) for 3,5-dichlorphenol (reference compound) was 5.7 mg/L, in accordance with the proposed guidance value in the protocol.

The same antibacterial agents as before was assessed with the pour plate method and showed to be five to eight times more potent with this method. EC_{50} (mg/L) was, in decreasing order: MET (449), SDZ (35.4), O (17.9), BP (10.3), TYL (6.1), TI (3.9), ST (0.17), OTC (0.14), TC (0.321), OXA (0.064), and CTC (0.028). Except for SDZ the antibacterial agents with a broad spectrum of activity were the most potent ones. The EC_{50} (mg/L) of 3,5-dichlorophenol (reference compound) was 6.0, and consistent with the guidance values in the ISO 15522 (ISO 1999) protocol. No guidance value exists for the pour plate method.

Inhibition of the Nitrifying Bacteria

Unexpected results were obtained with the nitrification inhibition test (ISO 1989). Even though tests were repeated three times it was only possible because of very broad 95% confidence limits on the EC values to assess the results as either stimulating or inhibiting the nitrification rate. Results showed, however, that OTC, CTC, TI, and ST inhibited the nitrification and that SDZ, OXA, O, and TYL stimulated the nitrification. Tests with TC were not performed. Phenol and 3,5-dichlorophenol (EC_{50} = 2.1 and 1.3 mg/L, respectively) were tested as reference compounds and comparison with literature values showed consistency with previous obtained values (Gernaey *et al.* 1997).

Growth inhibition test with *N. europaea* showed (as expected) that the antibacterial agents having a broad spectrum of activity (TC, CTC, OTC, ST) or affecting Gram-negative bacteria selectively (O) were the most potent ones. SDZ and TYL (Gram-positive) did not affect the nitrifying bacteria. The toxicity (EC_{50} value, mg/L) found was in decreasing EC_{50} values: TI (23.3), SDZ (17.0), TC (4.0), OTC (1.7), OXA (1.0), CTC (0.64), O (0.03), ST (0.02). NOECs of 100 and 50 mg/L were found for MET and TYL, respectively.

The tetracyclines (TC, CTC, and TC) and OXA were all potent to *N. europaea* tested with the pour plate method. Again this method was more sensitive (at least a factor 10 for the tetracyclines, less for OXA) than the previous described. EC_{50} (mg/L) values for TC, OTC, CTC, and OXA were found to be 0.2, 0.002, 0.3, and 0.5, respectively.

Comparison of Test with and Without Tween 80

Tests with and without the nonionic surfactant Tween 80 were performed with OTC and gave EC_{50}s (mg/L) of 1.24 and 1.51, respectively (ISO 15522 method [ISO 1999]). For TYL the corresponding results were 108 and 131, respectively. For 3,5-dichlorphenol (reference compound) 15.2 mg/L and 14.0 mg/L were found, respectively. Similar comparing tests using the pour plate method for OTC gave EC_{50}s (mg/L) of 0.15 and 0.18, respectively. For TYL 8.04 and 7.54, respectively, and for O 5.95 and 6.87, respectively. Figure 2 shows the statistical comparison of OXY with and without Tween 80 for both methods. No statistical difference was revealed between test with or without addition of 50 mg/L Tween 80 because the 95% confident limit of the two fitting parameters in the Weibull equation overlapped in each of the paired experiments.

Antibacterial-Resistant Sludge Bacteria

The activated sludge bacteria used in the tests showed resistance to all the tested antibacterial agents. At a concentration

Table 3. Result expressed as EC_{50} (mg/L) values for the antibacterial agents tested with corresponding 95% confidence limit

	Growth Inhibition of Sludge Bacteria		Inhibition of Nitrification		
Antibacterial Agents	ISO 15522 EC_{50} (mg/L)	Pour Plate Method with Sludge EC_{50} (mg/L)	ISO 9509 Nitrification Inhibition EC_{50} (mg/L) or Increased/ Decreased Nitrification Rate	Growth Inhibition *Nitrosomonas europaea* EC_{50} (mg/L)	Pour Plate Method with *Nitrosomonas europaea* EC_{50} (mg/L)
β-lactam antibiotics					
Benzylpenicillin (penicillin G) (BP)	84.6 [77.9–91.9]	10.3 [5.5–19.2]	NA	NA	NA
Tetracyclines					
Oxytetracycline (OTC)	1.2 [1.0–1.4]	0.14 [0.11–0.19]	↓	1.71 [0.55–5.23]	0.32 [0.28–0.34]
Chlortetracycline (CTC)	0.4 [0.2–0.9]	0.028 [0.023–0.034]	↓	0.64 [0.30–1.37]	0.002 [0.0018–0.0033]
Tetracycline (TC)	2.2 [1.2–4.3]	0.321 [0.263–0.392]	NA	4.0 [3.2–4.9]	0.20 [0.14–0.28]
Quinoxalin					
Olaquindox (O)	95.7 [82.1–108.3]	17.9 [11.0–29.2]	↑	0.031 [0.009–0.067]	NA
Aminoglucoside					
Streptomycin (ST)	0.47 [0.24–0.91]	0.17 [0.15–0.20]	↓	0.016 [0.003–0.123]	NA
Pleuromutilin derivative					
Tiamulin (TI)	14.3 [11.6–17.8]	3.9 [2.3–6.5]	↓	23.3 [16.1–33.8]	NA
Macrolide					
Tylosin (TYL)	54.7 [44.6–67.0]	6.1 [3.5–10.7]	↑	NOEC = 50	NA
Quinolone					
Oxolinic acid (OXA)	0.10 [0.07–0.13]	0.064 [0.037–0.108]	↑	1.0 [0.8–1.5]	0.46 [0.38–0.58]
Sulfonamide					
Sulfadiazin (SDZ)	NOEC = 60	35.4 [21.7–57.8]	↑	17.0 [14.4–23.8]	NA
Imidazol					
Metronidazol (MET)	NOEC = 100	449 [385–523]	NA	NOEC = 100	NA
Reference compounds					
Phenol	NA	NA	2.1 [1.3–3.4]	NA	NA
3,5-dichlorphenol	5.7 [5.5–6.0]	6.0 [5.5–6.6]	1.31 [1.28–1.35]	NA	NA

↑ = Nitrification rate relatively increased compared to the controls.
↓ = Nitrification rate relatively reduced compared to the controls.
NA = not available

level of 8 mg/L oxytetracycline in the test still 4% of the bacterial colonies were developed compared to the controls. Bacterial colonies developed at that high a concentration level are considered to have developed resistance to tetracyclines (NCCLS 1997). Similar data for the other antibacterial agents were (antibacterial agent concentration, mg/L); CTC (8 mg/L, < 2%), TC (8 mg/L, < 2%), BP (400 mg/L, 16%), TYL (200 mg/L, 8%), ST (10 mg/L, 4%), SDZ (150 mg/L, 27%), OXA (1 mg/L, 17%), TI (100 mg/L, 8%). This shows that except for BP and SDZ almost 10% of the activated sludge bacteria were resistant (BP = 16% and SDZ = 27%) to the highest test concentration level applied in the test.

Discussion

The potency of 11 different antibacterial agents covering 9 different groups of antibacterial agents were tested for the inhibition of growth and nitrification rate of activated sludge bacteria using standardized methods. Up to 10% of the applied activated sludge bacteria appeared resistant to the applied antibacterial agents. For BP and SDZ this was even higher. Resistance development may probably affect the EC_{50} values because the tested agents has a lower potency on such bacteria.

Cross-resistance between antibacterial agents was not assessed. We considered that the activated sludge from the Lyngby STP was applicable in the current study because even though resistance was detected, it was not a major problem (< 10% resistance).

It is not possible with the data presented in Table 3 to generalize toxic relations among the antibacterial agents for any of the presented bacterial cultures. This is consistent with the results on *V. fischeri* presented by Backhaus and co-workers (Backhaus *et al.* 1997; Backhaus and Grimme 1999). On the other hand, it seems that antibacterial agents with a narrow spectrum of activity (*e.g.*, O, TYL, and MET), showed to be

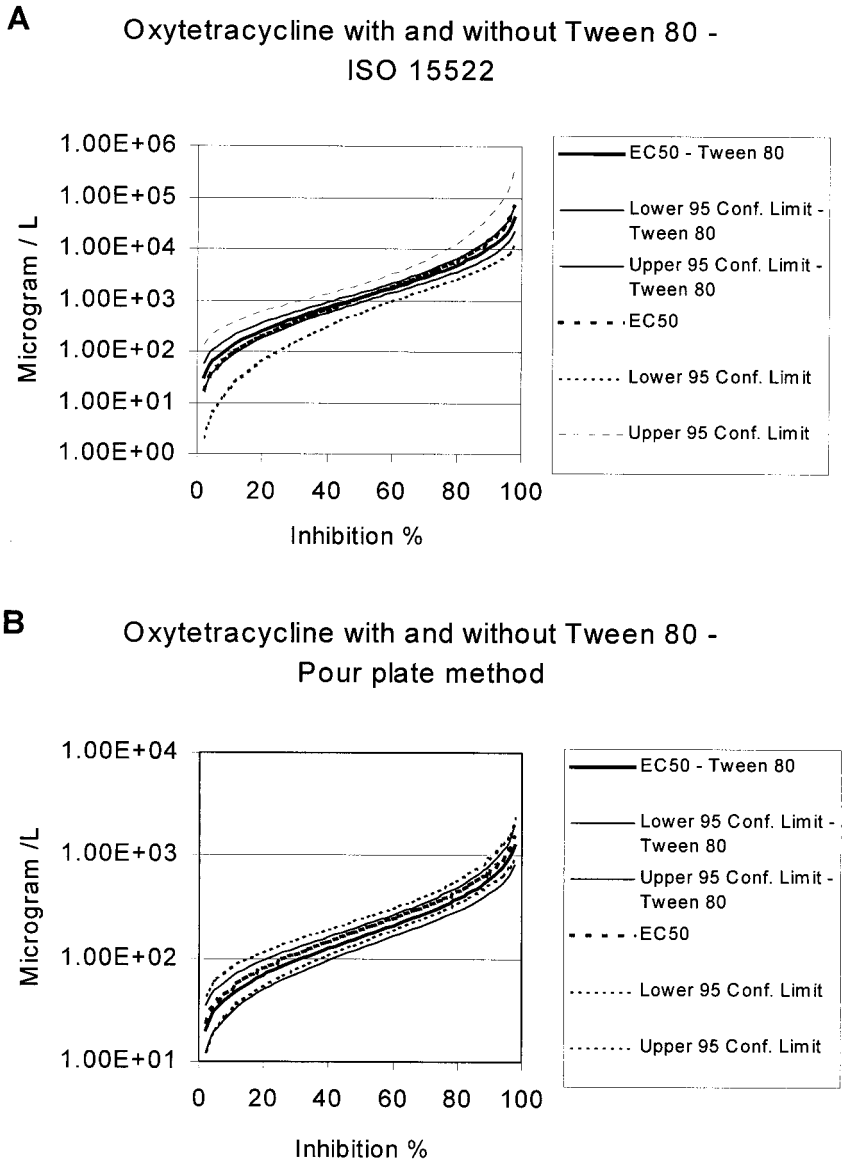

Fig. 2. Weibull dose-response relationships with corresponding upper and lower 95% confidence limits for the inhibition of activated sludge by OXY with and without Tween 80. (A) growth inhibition ISO 15522; (B) pour plate method

rather nonpotent (high EC_{50}s), compared to agents with a broad spectrum of activity (except SDZ) applying the ISO 15522 (ISO 1999) guideline. This suggests that antibacterial agents with a narrow spectrum of activity suppress only certain bacterial species in the sludge, enabling other species to grow faster. The overall inhibition picture will thus only be relative moderate compared with probable inhibition on vulnerable single bacterial species and may lead to the conclusion that the standardized methods should be used with care assessing the inhibition of antibacterial agents on bacteria. SDZ was the only antibiotic with a broad spectrum of activity having an NOEC value higher than 60 mg/L (NOECs were defined, as was the highest test concentration in the test not affecting the bacteria). The low potency of SDZ may be explained by their mode of action; sulfonamides inhibit the synthesis of folic acid. Bacteria have reserves of growth factors (*e.g.*, folic acid) that may prevent the effect of, *i.e.*, sulfonamides for several cell generations. The effect will therefore first be visible when reserves of folic acid have been depleted from the cells (Greenwood and O'Grady 1976). Both sludge and growth media may also contain folic acid or other growth constituents and may contribute to this. Short test duration (between 4 and 6 h) may therefore be the reason for the weak potency of SDZ in these tests. This is supported by the fact that no toxicity of SDZ was seen in the ISO 15522 (ISO 1999) test (NOEC = 60 mg/L) and only a low potency with the pour plate method (EC_{50} = 35.4 mg/L).

To encompass some of the previous indicated factors (*e.g.*, prolonged test duration and clumping of bacteria) I tried to assess the same antibacterial agents with a pour plate method with sludge bacteria. With this method the antibacterial agents were 5 to 10 times more potent than with the ISO 15522 (ISO 1999) method. Several factors may explain this. First, it is well know that bacteria may form clumps following exposure to certain antibacterial agents as reported by Sörén *et al.* (1995). Results obtained with a pour plate method are only valid if each single viable bacterium develops in to a colony. Otherwise, results will lead to an

overestimation of the potency. It is therefore suggested to add a nonionic surfactant, *e.g.*, Tween 80, in nontoxic concentrations to the test solution. Comparing tests with and without 50 mg/L Tween 80 (nontoxic to the sludge bacteria), were performed with OTC, TYL, and O on the same sludge sample. Statistically, there was no difference in the dose-response curves for the three antibacterial agents tested, indicating that Tween 80 had no influence (results for OTC are shown in Figure 2). The 5 to 10 times lower EC_{50} obtained on the pour plates could therefore not be accounted to clumping of bacteria. It is worth nothing that the reference compound 3,5-dichlorphenol exhibited an EC_{50} value in the same range in both the growth inhibition tests and the pour plate method. This suggests that the higher potency of the antibacterial agents in the pour plate method is related to better bioavailability of the compounds to the target bacteria in the bacterial community using this method. It was not easy to assess the inhibition of the nitrification rates with the ISO 9509 (ISO 1989) guideline. Dose-response relationships were not obtainable even though the experiments were repeated three times. Therefore it was only possible to evaluate the results of the tests as either stimulating or inhibiting the nitrification rate. The problem was probably not the test performance, as we obtained sound results for two reference compounds, phenol and 3,5-dichlorphenol (see Table 3). A nitrification rate of between 2 and 6.5 mg ($N\ g^{-1}\ h^{-1}$) was obtained in all experiments, as suggested by the guideline. It is therefore impossible to explain these results, but it is worthwhile to note that ST, which selectively affects Gram-negative bacteria, reduced the nitrification rate. Convorsely and impossible to explain, both OXA and O (both affecting Gram-negative) stimulated the nitrification rate. Furthermore, and also not obvious, all the broad-spectrum antibacterial agents (except OTC and CTC) stimulated the nitrification rate.

Because results with the ISO 9509 (ISO 1989) test were not obvious, I also assessed the same antibacterial agents on a strain of *N. europaea*. The endpoint applied here was the same as used for sludge bacteria in the ISO 15522 (ISO 1999) test. ST and O, both selectively affecting Gram-negative bacteria, were highly potent with EC_{50}s in the μg/L range, whereas the broad-spectrum antibacterial agents were less potent with EC_{50}s in the mg/L range. Furthermore I applied the same pour plate method as before on the nitrifying bacteria. Even though only four compounds was assessed (OTC, CTC, TC, and OXA) again the pour plate method showed at least a factor 10 times more potent (tetracyclines) compared to the growth inhibition test.

Conclusion

It was not possible to generalize toxic relations among the antibacterial agents for any of the presented bacterial cultures. But it should be noticed that EC_{50}s in the μg/L range were observed for several of the agents with a broad spectrum of activity. Agents with a more narrow spectrum of activity exhibited EC_{50}s in the mg/L range. The long-term inhibition test using pour plate method with sludge bacteria or *N. europeae* seems to be especially sensitive to most of the antibacterial agents compared to standardized tests. The pour plate method was found to be at least a factor of 10 more potent for all antibacterial agents except SDZ, TI, and MET. The nitrification rate was assessed with the ISO 9509 (ISO 1989) method. Even though results for two positive controls was found in accordance with the literature, the test was not easy to perform with the antibacterial agents. Triplicate tests only gave indications on the level of increased or decreased nitrification rate. More work should be done to assess the effects of antibacterial agents on the nitrogen cycle because available methods seems difficult to perform with such compounds. More accurate data for the inhibition of *N. europaea* was found with both a suspended culture and a similar pour plate method as for the heterotrophs.

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References

ACD (1995) User's manual. Advanced Chemistry Development, Toronto, Ontario, Canada

Andersen JS, Holst H, Spliid H, Andersen H, Nyholm N, Baun A (1998) Continuous ecotoxicological data evaluated relative to a control response. J Agric Biol Environ Stat 3:405–420

Backhaus T, Grimme LH (1999) The toxicity of antibiotic agents to the luminescent bacterium "*Vibrio fischeri*". Chemosphere 38(14): 3291–3301

Backhaus T, Froehner K, Altenburger R, Grimme LH (1997) Toxicity testing with *Vibrio fischeri*: a comparison between the long term (24 h) and short term (30 min) bioassay. Chemosphere 35(12): 2925–2938

Bird AE, Marchall AC (1967) Correlation of serum binding of penicillins with partition coefficients. Biochem Pharmacol 16:2275–2290

Bird AE, Marchall AC (1971) Reversed-phase thin-layer chromatography and partiotioning coefficients of penicillins. J Chromatogr 63:313–319

Colaizzi J, Klink P (1969) pH-partitioning behavior of tetracyclines. J Pharm Sci 58:1184–1189

Danish Standard (1983) DS 2251—Vandundersøgelse. Bestemmelse af aerobt kimtal ved 21°C. 1. udgave pp 195–198 (in Danish)

Gernaey K, Verschuere L, Luyten L, Verstrate W (1997) Fast and sensitive acute toxicity detection with an enrichment nitrifying culture. Water Environ Res 69(6):1163–69

Greenwood D, O'Grady F (1976) Activity and interaction of trimethoprim and sulphamethoxazole against *Escherichia coli*. J Clini Pathol 29:162–166

Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE (1998) Occurence, fate and effects of pharmaceuticals in the environment—a review. Chemosphere 36(2):357–393

Herbert BJ, Dorsey JG (1995) n-Octanol-water partition coefficient estimation by micillar electrokinetic capillary chromatography. Anal Chem 67:744–749

Holten Lützhøft HC, Halling-Sørensen B, Jørgensen SE (1999) Algal toxicity of antibacterial agent applied in Danish fish farming. Arch Environ Contam Toxicol 36:1–6

ISO (1984a) 7150/1 Water quality—determination of ammonium—Part 1: Manual spectrophotometric method. ISO, Switzerland

ISO (1984b) 6777 Water quality—determination of nitrit—molecular absorption spectrophotometric method. ISO, Switzerland

ISO (1986) 7890/1 Water quality—determination of nitrate—Part 1: 2.6-Dimethylphenol spectrophotometric method. ISO, Switzerland

ISO (1989) 9509 Method for assessing the inhibition of nitrification of activated sludge micro-organisms by chemicals and waste waters. ISO, Switzerland

ISO (1999) 15522 Determination of the inhibitory effect of water constituents on the growth of activated sludge micro-organisms. ISO, Switzerland

Jørgensen SE, Halling-Sørensen B (2000) Editorial—drugs in the environment. Chemosphere 40(7):691–699

Koizumi T, Arita T, Kakemi K (1964) Absorption and excretion of drugs. XIX. Some pharmakokinetics aspects of absorption and excretion of sulphonamides. Absorption from rat stomach. Chem Pharm Bull 12:413–420

Lund W (ed) (1994) The pharmaceutical codex, principles and practise of pharmaceuticals, 12th ed. Pharmaceutical Press, London

McFarland JW, Berger CM, Froshauer SA, Hayashi SF, Hecker SJ, Jaynes BH, Jefson MR, Kamicker BJ, Lipinski CA, Lundy KM, Reese CP, Vu CB (1997) Quatitative structure—activity relationship among macrolide antibacterial agents: *in vitro* and *in vivo* potency against *Pasteurella multocida*. J Med Chem 40:1340–1346

Merck (1998) Merck veterinary manual, 8th ed. Edited by Merck & Co.

Miller GH, Smith HL, Rock WL, Hedberg S (1977) Antibacterial structure-activity relationship obtained with resistant microorganisms. 1 Inhibition of R-factor resistant *Escherichia coli* by tetracyclines. J Pharm Sci 66:88–92

Mow T, Thøgersen C (1997) Veterinært farmakologikompendium. DSR Forlag, Acartiavej 7, Frederiksberg, Danmark (in Danish)

NCCSL (1997) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically—4th ed.; approved standard. NCCLS document M7-A4, National Committee of Clinical Laboratory Standards, Wayne, PA

Parke TV, Davis WW (1954) Use of apparent dissociation constants in qualitative organic analysis. Anal Chem 26:642–645

Rapson HDC, Bird AE (1963) Ionisation constants of some penicillins and of their alkaline and penicillinase hydrolysis products. J Pharm Pharmacol 15:222T–231T

Ryrfeldt A (1971) Biliary excretion of penicillins in the rate. J Pharm Pharmacol 23(6):463–464

Sayed AM, Ibrahim RS, Aziz AMA (1998) Antimicrobial in-vitro sensitivity testing of *Salmonella emele* and *E. coli* isolates using minimum inhibitory concentration (MIC). Assint Veterinary Medical Journal 40:73–82.

Stephens CR, Murai K, Brunings KJ, Woodward PB (1956) Acidity constants of the tetracyclines antibiotics. J Am Chem Soc 78: 4155–4158

Sörén L, Nilsson M, Nilsson LE (1995) Quantitation of antibiotic effects on bacteria by bioluminescence, viable counting and quantal analysis. J Antimic Chemother 35:669–74

Takács-Novák K, Józan M, Hermecz I, Szász G (1992) Lipophilicity of antibacterial flouroquinolones. Int J Pharm 79:89–96

Timmers K, Sternglanz R (1978) Ionization and divant cation dissociation constants of nalidixic and oxolinic acids. Bioinorg Chem 9:145–155

Ziv G (1980) Preliminary clinical pharmacological investigations of tylosin and tiamulin in chickens. Veterinary Quarterly 2:206–210