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Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics

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ABSTRACT

Antibiotics may affect both primary producers and decomposers, potentially disrupting ecosystem processes. Hence, it is essential to assess the impact of antibiotics on aquatic ecosystems. The aim of the present study was therefore to evaluate the potential of a recently developed test for detecting antibiotics in animal tissue, the Nouws Antibiotic Test (NAT), as a sensitive bioassay to assess the effects of antibiotics in water. To this purpose, we determined the toxicity of sulphamethoxazole, trimethoprim, flumequine, tylosin, streptomycin, and oxytetracycline, using the NAT adapted for water exposure. The sensitivity of the NAT was compared to that of bioassays with bacteria (Microtox), cyanobacteria and green algae. In the Microtox test with *Vibrio fischeri* as test organism, no effects were observed for any of the test compounds. For three of the six antibiotics tested, the cyanobacteria were more vulnerable than the green algae when using photosynthetic efficiency as an endpoint. The lowest EC50 values for four out of six tested antibiotics were obtained using the NAT bacterial bioassay. The bacterial plate system responded to antibiotics at concentrations in the μg L^{-1} and lower mg L^{-1} range and, moreover, each plate proved to be specifically sensitive to the antibiotics group it was designed for. It is concluded that the NAT bioassay adapted for water exposure is a sensitive test to determine the presence of antibiotics in water. The ability of this test to distinguish five major antibiotic groups is a very strong additional value.

1. Introduction

Antibiotics are widely used to treat infections in humans and are applied intensively for veterinary purposes. Because they are poorly metabolized in the body (Christensen, 1998; Andreozzi et al., 2006; Vieno et al., 2006) and incompletely degraded in wastewater treatment plants (Lanzky and Halling-Sørensen, 1997; Hartmann et al., 1998; Arslan-Alaton and Caglayan, 2006), antibiotics are continuously introduced into the environment. Consequently, in spite of their relatively short environmental half-lives, they are ubiquitous in aquatic environments.

In addition to the human health risks of the presence of an increasing amount of resistant bacteria in the environment (Kim and Aga, 2007; Kümmerer, 2009), and the unwanted presence of antibiotics in drinking water (Zuccato et al., 2000; Ye et al., 2007), there is a growing concern for the ecological risk of antibiotics in the aquatic environment. Antibiotics are specifically applied to fight pathogenic bacteria, but in the environment non-target organisms are inevitably exposed (Flaherty and Dodson, 2005), resulting in a potential risk of negative effects on indigenous microorganisms. These non-target microorganisms provide important ecosystem services, such as nutrient cycling, organic matter mineralization and degradation of pollutants (Näslund et al., 2008). But also primary producers, like microalgae and especially cyanobacteria, being prokaryotes, may be vulnerable to antibiotics (Cabello, 2006; Maul et al., 2006). Thus, antibiotics may affect both primary producers and decomposers, potentially disrupting ecosystem processes. Therefore, it is essential to monitor and assess the impact of antibiotics on aquatic ecosystems.

Water quality monitoring has historically relied heavily on chemical analyses. Such measurements may identify compounds present in the environment, but do not give insight in the bioavailability of the present toxicants and the joint effects of mixtures of (un)known compounds on biota (Hendriks et al., 1994). Therefore, bioassays are deployed as a complementary tool, giving insight in biological effects. Ideally, but seldomly achieved, bioassays should also indicate the group of compounds responsible for the observed effect. Since bacteria are the target organisms for antibiotics, we

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expected that toxicity assessment with bacteria would provide a sensitive method to determine the effects of antibiotics in the aquatic environment. Yet, the classical acute Microtox test system, with a marine bacterium (*Vibrio fischeri*) as test organism, has not proven very sensitive to antibiotics (Ferrari et al., 2004; Isidori et al., 2005; Christensen et al., 2006). Alternatively, cyanobacteria could be used as test organisms and it has indeed been found that cyanobacteria were up to two orders more sensitive to antibiotics than green algae (Holten-Lützhøft et al., 1999; Halling-Sørensen, 2000). These classical algal growth toxicity tests are however not rapid screening tools and are not capable of identifying different groups of antibiotics. Alternatively, algal toxicity testing can also be performed with photosynthetic efficiency as acute endpoint instead of growth inhibition as chronic endpoint (e.g. Drábková et al., 2007a), making the test less time consuming, but still not capable of identifying the causing group of agents.

The aim of the present study was therefore to evaluate the potential of a recently developed test for detecting antibiotics in animal tissue, the Nouws Antibiotic Test (NAT, Pikkemaat et al., 2008), as a sensitive bioassay to determine the effects of antibiotics in water. The ability of this method to distinguish five major antibiotic groups may prove a very strong additional value. We compared the sensitivity of the NAT test to bioassays with a green alga and with a cyanobacterium using photosynthetic efficiency as an endpoint, and with the classic acute Microtox test using bacteria.

2. Materials and methods

2.1. Test compounds

Test compounds were chosen to represent different classes of antibiotics. Evidence of occurrence in surface water (Hirsch et al., 1999; Hilton and Thomas, 2003; Schrap et al., 2003) and stability and solubility were additional criteria for selection. The following antibiotics were selected: sulphamethoxazole, trimethoprim and flumequine (ICN Biomedicals Inc., Aurora, OH, USA), tylosin tartate and streptomycin sulfate (Sigma–Aldrich, Zwijndrecht, The Netherlands), and oxytetracycline dihydrate (Arcos Organics, Fisher Emergo BV, Landsmeer, The Netherlands).

Sulphamethoxazole (a sulphonamide) is a folate antagonist, blocking the conversion of *p*-aminobenzoic acid to the coenzyme dihydrofolic acid in microorganisms. Trimethoprim also interferes with folate synthesis in susceptible bacteria by binding to, and reversibly inhibiting the enzyme dihydrofolate reductase. Sulphamethoxazole and trimethoprim are often prescribed together because of their synergistic effect. They are broad-spectrum antibiotics and active against gram negative and gram positive bacteria (Sweetman, 2002). Flumequine (a fluoroquinolone) inhibits the bacterial DNA gyrase, which prevents DNA replication; it is most effective against gram-negative bacteria (Rang et al., 1995). Tylosin (a macrolide) is a veterinary antibiotic. Macrolides exert their antibiotic effects by binding irreversibly to the 50S subunit of bacterial ribosomes, inhibiting translocation of tRNA during translation (the production of proteins under the direction of DNA). This action is mainly bacteriostatic, meaning that bacterial growth and reproduction are inhibited, in contrast to bactericidal antibiotics which directly kill bacteria. Tylosin has a narrow working spectrum and is active against gram positive bacteria (Sweetman, 2002). Streptomycin (an aminoglycoside) irreversibly binds to the bacterial 30S ribosome, freezing the 30S initiation complex (30S-mRNA–RNA) so that no further initiation can occur. It also slows down protein synthesis that was already initiated and induces misreading of the mRNA (Sweetman, 2002). Oxytetracycline (a tetracycline) is a broad-spectrum antibiotic that inhibits bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome (Sweetman, 2002).

The antibiotics were dissolved in Milli-Q water prior to testing and were diluted with the appropriate medium immediately before the tests. Twelve (NAT test), 10 (algal tests) and four (Microtox® test) concentrations per compound were tested, ranging from 0.1 $\mu g\ L^{-1}$ to 10 $mg\ L^{-1}$ (ranges were smaller when solubility of the compounds was low). Medium without compounds was used as a control. In all biological tests, we referred to nominal (=initial) antibiotic concentrations.

2.2. Bioassays with algae and cyanobacteria

The green alga *Pseudokirchneriella subcapitata* (SKULBERG1959/1, CCALA433, formerly known as *Selenastrum capricornutum* or *Raphidocelis subcapitata*) was cultivated in a continuous culture on Woods Hole medium (Guillard and Lorenzen, 1972). The culture was continuously aerated (50 $L\ h^{-1}$) in a 1 L chemostat at 20 °C and permanently illuminated with two circleline TL tubes (32W cool white). Two weeks prior to the experiments, algae were transferred to a batch culture and replenished with fresh medium every 2 d to adapt to static conditions. The cyanobacterium *Microcystis aeruginosa* (PCC 7806) was cultivated in Erlenmeyer flasks at 20 °C, an irradiance of 10 $\mu mol\ m^{-2}\ s^{-1}$, and a light/dark cycle of 16 h/8 h for 3–5 d to achieve exponential growth phase.

At the start of the toxicity tests, the algal cultures were diluted with fresh medium to achieve an initial cell density of 3×10^6 mL^{-1} for *P. subcapitata* and 10×10^6 mL^{-1} for *M. aeruginosa*. These cell densities were the lowest possible for reliable measurements with pulse amplitude modulated (PAM) fluorometry (see below) and represent approximately equal biomass levels for each of the two test species. Cell densities were verified using a Bürker counting chamber.

Toxicity tests were performed in transparent polystyrol flatbottom 96-well microplates (Greiner, Bio One BV, Alphen ad Rijn, The Netherlands). Per well, a volume of 100 μL of algal suspension, diluted with 200 μL Dutch Standard Water (DSW) (NEN, 1980) with or without antibiotics was used. Each concentration of antibiotics was tested in triplicate, but for the controls six replicates were tested. The tests were run for 24 h at 20 °C at an irradiance of 30 $\mu mol\ m^{-2}\ s^{-1}$ for *P. subcapitata* and 10 $\mu mol\ m^{-2}\ s^{-1}$ for *M. aeruginosa*.

After 24 h of exposure, the maximal yield of photosystem II was determined by PAM fluorescence, using a PAM-CONTROL fluorometer (Heinz Waltz GmbH, Germany), as in Drábková et al. (2007a). The cells were dark-adapted for 30 min before measurements. The parameters measured were: F_0, the minimal fluorescence signal of dark-adapted cells and F_m, the maximal signal of dark-adapted cells obtained with a saturating light pulse. These parameters allow the calculation of the maximal yield of photosystem II (F_v/F_m or $(F_m - F_0)/F_m$) which indicates the capacity of dark-adapted cells to convert light energy into chemical energy. This nomenclature is according to Van Kooten and Snel (1990). F_v/F_m is biomass independent and can be used as an indicator for the fitness of photosynthetic organisms. Control yield (F_v/F_m) was: 0.21 ± 0.04 (n = 36) for *M. aeruginosa* and 0.62 ± 0.02 (n = 36) for *P. subcapitata*, in agreement with values reported by Drábková et al. (2007b). Atrazine was included as positive control.

2.3. Bioassays with bacteria

The Microtox® test system measures the decrease in light output of the luminescent marine bacterium *V. fischeri*. Toxicants affecting the metabolism of the bacterium reduce luminescence, which was measured after 30 min and compared to the control (Bulich, 1979). Phenol was included as positive control.

The Nouws Antibiotic Test (NAT) for detecting veterinary drug residues (Pikkemaat et al., 2008) was adjusted for analysis of water samples. The test comprises a medium inoculated with a bacterium susceptible to a specific group of antibiotics and relies on agar diffusion of the antibiotic compound, with bacterial growth as endpoint. The original test consists of five plates which provide a group-specific identification: the T-plate for detection of tetracyclines, the Q-plate for detection of quinolones, the B&M-plate for detection of ß-lactams and macrolides (including lincosamides and pleuromutilins), the S-plate for the detection of sulphonamides and the A-plate for detection of aminoglycosides. For the analysis of water samples, the original format of the test, an agar layer in a square 120 × 120 mm petridish comprising nine sample holes, was converted to a microtiter format. The A-plate composition, however, proved to be incompatible with this change of format (the microorganism appeared not to be viable under the applied conditions), so the A-plate was omitted from the test. Basic media containing 31.4 g L^{-1} Iso-sensitest Agar (Oxoid) (T-plate and B&M-plate), 15.7 g L^{-1} Plate Count Agar (Difco) + 1% of a 1-M phosphate buffer pH 6.5 (Q-plate) and 40 g L^{-1} Diagnostic Sensitivity Test Agar (Oxoid) (S-plate) were sterilized for 15 min. After cooling down to 48 °C the T-plate was prepared by adjusting pH of the agar to 6.0, adding 500 μg L^{-1} chloramphenicol and inoculating the agar with 10^5 CFU mL^{-1} *Bacillus cereus* ATCC 11778. The Q-plate was prepared by inoculating the agar with 10^6 CFU mL^{-1} *Yersinia ruckeri* NCIMB 13282. The B&M-plate was prepared by adjusting the pH to 8.0, adding 7.5 μg L^{-1} tylosin and inoculating 10^6 CFU mL^{-1} *Micrococcus luteus* ATCC 9341. The S-plate was prepared by adjusting the pH to 7.0, adding 7 μg L^{-1} trimethoprim and inoculating with 10^6 CFU mL^{-1} *Bacillus pumilus* CN 607. Inoculated agars (200 μL per well) were pipetted in a flatbottom transparent polystyrol 96-well plate (Greiner bio One BV, Alphen ad Rijn, The Netherlands). After solidification of the agar, a dilution series of each test compound was added to a plate-specific buffer (1 M phosphate buffers at pH 6.0 (T-plate), pH 6.5 (Q-plate), pH 8.0 (B&M-plate) and a 0.5 M phosphate buffer pH 8.0 for the S-plate) in a ratio of 1:9, and directly applied onto the agar (70 μL of sample in buffer) in duplicate. At the start of the experiment the optical density (OD 600 nm) was read using a plate reader, and the plates were incubated at the appropriate temperature (T-, Q- and M-plate at 30 °C and S-plate at 37 °C). After 24 h of exposure, the wells were rinsed with water to eliminate contamination by fungi and bacteria growing on top of the agar, and the optical density (600 nm) of the agar was measured. Change in OD (ΔOD) was calculated by subtracting the initial OD from the OD at the end of the experiment. Neomycin, flumequine, erythromycin, sulphamethoxazole and oxytetracycline were included as positive control.

2.4. Data analysis

Inhibition of photosynthetic yield (F_v/F_m) and inhibition of growth (ΔOD) were expressed as percentage of the corresponding controls and plotted against the nominal antibiotic concentrations in the water. EC50 values (50% inhibition concentration) and their corresponding 95% confidence limits were calculated by a nonlinear curve-fitting procedure in GraphPad 5 program using the log vs. response with variable slope model: Y = Bottom + (Top–Bottom)/(1 + 10^((Log EC50 − X) * HillSlope)) in which Y is the

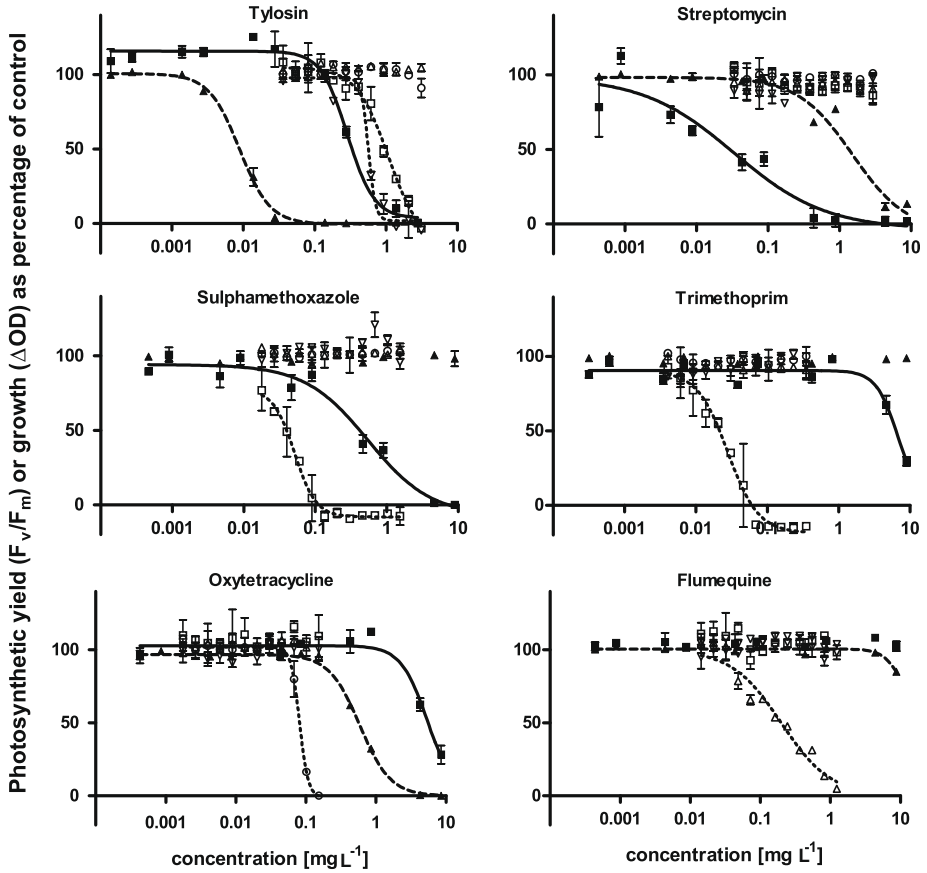

Fig. 1. Inhibition of photosynthetic yield (F_v/F_m) measured in the algal tests (closed symbols) and inhibition of growth (ΔOD) measured in the bacterial tests (open symbols) after 24 h exposure, expressed as mean percentage of the corresponding controls with standard deviation, plotted against the nominal antibiotic concentrations (mg L^{-1}). Hatched lines: response curves for *Pseudokirchneriella subcapitata*, solid lines: response curves for *Microcystis aeruginosa*, dotted lines: response curves for the bacteria; ○: bacteria on T-plate, □: bacteria on S-plate, Δ: bacteria on Q-plate, ▽: bacteria on M-plate, ■: cyanobacterium, ▲: green alga.

inhibition (% of control value), HillSlope is the slope, and X is log concentration (mg L^{-1}).

3. Results

In the acute Microtox test the positive control showed inhibition, but no effect was observed for any of the antibiotics in the tested concentration range (results not shown). In the test with the green alga clear concentration response relationships were obtained for four of the six tested compounds (Fig. 1). In the cyanobacterial test and in the Nouws Antibiotic Test this was the case for five of the six compounds. From the concentration–response curves median effective concentrations (EC50) and the corresponding 95% confidence intervals were derived (Table 1).

For trimethoprim, sulphamethoxazole, oxytetracycline and flumequine, the lowest observed EC50 values (0.028, 0.052, 0.081 and 0.2 mg L^{-1} respectively) were obtained in the bacterial plate tests. These EC50 values were found on the plate sensitive to the antibiotic group to which the tested antibiotic belonged: trimethoprim and sulphamethoxazole (a di-amino pyrimidine and a sulphonamide) on the S-plate, oxytetracycline (a tetracycline) on the T-plate and flumequine (a fluoroquinolone) on the Q-plate. The M-plate was the most sensitive bacterial plate to tylosin, a macrolide (EC50 of 0.57 mg L^{-1}). The S-plate also responded to tylosin, but with a higher EC50 (Fig. 1). The lowest observed EC50 (0.034 mg L^{-1}) for streptomycin was found for the cyanobacteria.

The overall lowest observed EC50 (0.0089 mg L^{-1}) was found for the green algae exposed to tylosin. In our study we did not observe a generally higher sensitivity of cyanobacteria to the tested antibiotics compared to the green algae, since for oxytetracycline and tylosin the green algae (EC50 values of 0.6 and 0.089 mg L^{-1} respectively) were more sensitive than the cyanobacteria (EC50 values of 5.4 and 0.29 mg L^{-1} respectively).

Overall toxicity of the tested antibiotics in increasing order of EC50 value was: tylosin (0.0089 mg L^{-1} for green algae), trimethoprim (0.028 mg L^{-1} on S-plate), streptomycin (0.034 mg L^{-1} for cyanobacteria), sulphamethoxazole (0.052 mg L^{-1} on S-plate), oxytetracycline (0.081 mg L^{-1} on T-plate) and flumequine (0.2 mg L^{-1} on Q-plate).

4. Discussion

Because of their prokaryotic nature, bacteria were expected to be the most sensitive to antibiotics. Yet, in the classical acute Microtox test with *V. fischeri* as test organism, no effects were observed for any of the test compounds after 30 min of exposure. This is in agreement with previous studies applying the Microtox test (Ferrari et al., 2004; Isidori et al., 2005; Christensen et al., 2006) and is most likely due to the very short exposure time (15–30 min) rather than to insensitivity of *V. fischeri* to antibiotics. This is confirmed by Thomulka et al. (1993) who reported substantial effects of several antibiotics on reproduction of the microbe *Vibrio harveyi* after 5 h of exposure, but obtained little evidence for short-term effects on bioluminescence. Similar time-dependent effects of antibiotics were found for *V. fischeri* in the Microtox test (Backhaus and Grimme, 1999; Froehner et al., 2000). Hence applying the Microtox tests to assess the effects of antibiotics would require a much longer exposure period than the prescribed 30 min. By doing so, reliable effect concentrations may be obtained, closer to the ones for the other tests species.

Alternatively, cyanobacteria could be used as test organisms, since it has been found that cyanobacteria were up to two orders more sensitive to antibiotics than green algae (Holten-Lützhøft et al., 1999; Halling-Sørensen, 2000). These classical algal growth toxicity tests are however not rapid screening tools, lasting for 3 d (green algae) or 7 d (cyanobacteria). To decrease exposure time, in the present study photosynthetic efficiency was measured as acute endpoint after 24 h of exposure. Below we will compare the sensitivity of the two methods for both green algae and cyanobacteria and will evaluate if cyanobacteria are still more sensitive to antibiotics than green algae when photosynthetic efficiency after 24 h of exposure is used as acute endpoint instead of growth as chronic endpoint after 3 or 7 d of exposure.

For tylosin, reported $EC50_{growth}$ values for *P. subcapitata* are up to 140 times higher than effect concentrations calculated in the present study using photosynthetic efficiency as acute endpoint (Table 2). This suggests that for this antibiotic, photosynthesis is more sensitive than growth. This is remarkable, since none of the tested antibiotics was designed to specifically affect photosynthesis (see Section 2.1). For trimethoprim, relatively high EC50 values were observed (Table 2), confirming the low sensitivity of *P. subcapitata* to this antibiotic, measured with different endpoints. For sulphamethoxazole, $EC50_{growth}$ values are one to two orders of magnitude lower than for the photosynthetic endpoints determined in the present study (Table 2), suggesting that expression of effects of this antibiotic requires more time, or that photosynthesis of *P. subcapitata* is not as susceptible to sulphamethoxazole as growth, as expected considering the mode of action of the antibiotics. Likewise, for streptomycin, the present EC50 value is 10 times higher than the reported $EC50_{growth}$ value (Table 2). For oxytetracycline, the present EC50 value is in the range of EC50 values found after 3 d (Table 2). For flumequine, we only observed the onset of inhibition at the tested concentration range, which is above reported EC50 values (Table 2). Thus only for tylosin, photosynthetic efficiency was a much more sensitive acute endpoint, while

Table 1
EC50 values (mg L^{-1} [95% CI]) for the six tested antibiotics obtained in the tests with four bacterial plates specifically sensitive to tetracyclines (T), sulphonamides (S), quinolones (Q) and macrolides (M) derived from growth inhibition (ΔOD) response curves, the acute Microtox test derived from inhibition of luminescence, and the tests with green algae (*Pseudokirchneriella subcapitata*) and cyanobacteria (*Microcystis aeruginosa*) derived from inhibition of photosynthetic yield (F_v/F_m) response curves. >: EC50 above the highest test concentration.

	Bact. (T)	Bact. (S)	Bact. (Q)	Bact. (M)	Microtox	*P. subcapitata*	*M. aeruginosa*
Tylosin	>3.1	1.09 [0.80–1.48]	>3.1	0.57 [0.53–0.61]	>1.8	0.0089 [0.0080–0.0098]	0.29 [0.25–0.33]
Trimethoprim	>0.35	0.028 [0.022–0.035]	>0.35	>0.35	>0.28	>9.0	6.9 [6.2–7.7]
Sulphamethoxazole	>1.5	0.052 [0.043–0.063]	>1.5	>1.5	>1.5	>9.0	0.55 [0.35–0.84]
Streptomycin	>2.9	>2.9	>2.9	>2.9	>0.4	1.5 [0.66–3.5]	0.034 [0.013–0.087]
Oxytetracycline	0.081 [0.077–0.086]	>0.15	>0.15	>0.15	>0.1	0.60 [0.56–0.62]	5.4 [4.6–6.4]
Flumequine	>1.2	>1.2	0.20 [0.17–0.24]	>1.2	>0.8	≈16	>8.8

Table 2
Comparison of EC50 values (mg L^{-1}; 95% CI between brackets) reported in literature and obtained in the present study. Effect concentrations are listed for the cyanobacterium *Microcystis aeruginosa* and the green alga *Pseudokirchneriella subcapitata* for the six tested antibiotics. Expo = exposure time (d).

Antibiotic	*Microcystis aeruginosa*	Expo (d)	*Pseudokirchneriella subcapitata*	Expo (d)	Reference
Tylosin	0.29 (0.25–0.33)	1	0.0089 (0.0080–0.0098	1	Present study
			0.411 (0.387–0.437)	3	Eguchi et al. (2004)
			0.21 (nd)	3	Yang et al. (2008)
	0.034 (0.024–0.048)	7	1.38 (0.97–1.96)	3	Halling-Sørensen (2000)
Trimethoprim	6.9 (6.2–7.7)	1	>9	1	Present study
	150 (nd)	7			Ando et al. (2007)
			80.3 (74.4–86.7)	3	Eguchi et al. (2004)
			40 (nd)	3	Yang et al. (2008)
	112 (100–126)	7	110 (64–192)	3	Halling-Sørensen et al. (2000)
	112 (100–126)	7	130 (81–211)	3	Holten-Lützhøft et al. (1999)
Sulphamethoxazole	0.55 (0.35–0.84)	1	>9	1	Present study
			0.146 (nd)	4	Ferrari et al. (2004)
			1.53 (1.40–1.68)	3	Eguchi et al. (2004)
			1.9 (nd)	3	Yang et al. (2008)
			0.52 (0.36–0.74)	3	Isidori et al. (2005)
Streptomycin	0.034 (0.013–0.087)	1	1.5 (0.66–3.5)	1	Present study
	0.007 (0.006–0.008)	7	0.133 (0.034–0.357)	3	Halling-Sørensen (2000)
Oxytetracycline	5.4 (4.6–6.4)	1	0.6 (0.56–0.62)	1	Present study
	0.23 (nd)	7			Ando et al. (2007)
			0.342 (0.321–0.364)	3	Eguchi et al. (2004)
			0.17 (0.11–0.25)	3	Isidori et al. (2005)
	0.207 (0.175–0.246)	7	4.5 (2.3–8.6)	3	Holten-Lützhøft et al. (1999)
Flumequine	>8.8	1	~16	1	Present study
	1.96 (1.76–2.16)	5	5 (4.8–5.2)	3	Robinson et al. (2005)
	0.159 (0.066–0.382)	7	5 (1.6–16)	3	Holten-Lützhøft et al. (1999)

for other antibiotics (sulphamethoxazole), the classical chronic method using growth after 3 d of exposure was much more sensitive, in line with the mode of action of the antibiotics (see Section 2.1).

Using the cyanobacterium *M. aeruginosa* as test organism for tylosin, streptomycin, flumequine and oxytetracycline, a one order of magnitude lower EC50 value was observed after 7 d in the classical growth test than in the present study (Table 2). For sulphamethoxazole, no literature data for *M. aeruginosa* are available. Thus, in contrast to the test with the green algae, the test with the cyanobacteria with an exposure time of 24 h and photosynthetic efficiency as acute endpoint systematically yielded higher EC50 values than the classical chronic growth test. This is in line with the mode of action of the antibiotics, not specifically designed to affect photosynthesis. In addition, the long exposure time (7 d) applied in the classical chronic growth test may have contributed to the low EC50 values reported in literature. This is confirmed by Robinson et al. (2005) applying an exposure time of 5 d instead of 7, resulting in an EC50 value of one order of magnitude higher than after 7 d exposure (1.96 and 0.159 mg L^{-1}). Thus applying photosynthesis as acute endpoint in cyanobacterial and green algae tests reduces exposure time, but often also sensitivity, not only due to the shorter exposure time, but also to the lack of specific effects of the antibiotics on photosynthesis.

Cyanobacteria are generally one to two orders of magnitude more sensitive than green algae when growth is measured as chronic endpoint (Holten-Lützhøft et al., 1999; Halling-Sørensen, 2000; Robinson et al., 2005). When photosynthetic efficiency after 24 h of exposure is used as acute endpoint however, only for three of the six antibiotics tested, the cyanobacteria were more vulnerable than the green algae (Table 2). Tylosin and oxytetracycline affected the green algae at concentrations one or two orders of magnitude lower than the cyanobacteria. Thus although cyanobacteria can be considered as target organisms for antibiotics, they are not always more sensitive to antibiotics than green algae.

Applying photosynthesis as acute endpoint in algal tests reduces exposure time, making the tests much more rapid, but nevertheless, algal tests are not capable of identifying different groups of antibiotics. The aim of the present study was therefore to evaluate the potential of a recently developed test for detecting antibiotics in poultry meat, the Nouws Antibiotic Test (NAT) (Pikkemaat et al., 2008), as a sensitive method to determine effects of antibiotics in water. In the present study, the lowest EC50 values for four out of six tested antibiotics were indeed obtained using the adapted NAT test, even though the agar constituents may have decreased the bioavailability of antibiotics. The bacterial plate system responded to antibiotics at concentrations in the μg L^{-1} and lower mg L^{-1} range and, moreover, each plate proved to be specifically sensitive to the antibiotics group it was designed for. Only for tylosin green algae were more sensitive than bacteria. For streptomycin, the sixth antibiotic tested, its specific plate (A-plate) could not be converted to a microtiter format. Considering the results reported for the original test format (Pikkemaat et al., 2008), it seems unlikely that a functional A-plate would be more sensitive than the EC50 of 0.034 mg L^{-1} found with the cyanobacteria in this study. For sulphamethoxazole, flumequine, trimethoprim and oxytetracycline enough data is available to compare the effect concentrations obtained in the present study with concentrations measured in the field (Hirsch et al., 1999; Hilton and Thomas, 2003; Schrap et al., 2003; Roberts and Thomas, 2006). This comparison reveals that environmental concentrations are in the μg L^{-1} range, while effects concentrations are generally in the higher μg L^{-1}, lower mg L^{-1} range. Yet, this may not hamper application of the adapted NAT test for monitoring the presence of antibiotics in field samples, since in routine monitoring programs in the Netherlands, water samples are concentrated up to a factor of 1000.

5. Conclusion

It is concluded that the NAT test adapted for water exposure is a sensitive test to determine the presence of antibiotics in water. The ability of this test to distinguish five major antibiotic groups is a very strong additional value.

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References

Ando, T., Nagase, H., Eguchi, K., Hirooka, T., Nakamura, T., Miyamoto, K., Hirata, K., 2007. A novel method using cyanobacteria for ecotoxicity test of veterinary antimicrobial agents. Environ. Toxicol. Chem. 26, 601–606.

Andreozzi, R., Canterino, M., Giudice, R.L., Marotta, R., Pinto, G., Pollio, A., 2006. Lincomycin solar photodegradation, algal toxicity and removal from wastewaters by means of ozonation. Water Res. 40, 630–638.

Arslan-Alaton, I., Caglayan, A.E., 2006. Toxicity and biodegradability assessment of raw and ozonated procaine penicillin G formulation effluent. Ecotoxicol. Environ. Saf. 63, 131–140.

Backhaus, T., Grimme, L.H., 1999. The toxicity of antibiotic agents to the luminescent bacterium *Vibrio fischeri*. Chemosphere 38, 3291–3301.

Bulich, A.A., 1979. Use of luminescent bacteria for determining toxicity in aquatic environments. ASTM STP 667, 98–106.

Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 8, 1137–1144.

Christensen, F.M., 1998. Pharmaceuticals in the environment – a human risk? Regul. Toxicol. Pharm. 28, 212–221.

Christensen, A.M., Ingerslev, F., Baun, A., 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. Environ. Toxicol. Chem. 25, 2208–2215.

Drábková, M., Admiraal, W., Maršálek, B., 2007a. Combined exposure to hydrogen peroxide and light – selective effects on cyanobacteria, green algae and diatoms. Environ. Sci. Technol. 41, 309–314.

Drábková, M., Matthijs, H.C.P., Admiraal, W., Marsalek, B., 2007b. Selective effects of H_2O_2 on cyanobacterial photosynthesis. Photosynthetica 45, 363–369.

Eguchi, K., Nagase, H., Ozawa, M., Endoh, Y.S., Goto, K., Hirata, K., Miyamoto, K., Yoshimura, H., 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57, 1733–1738.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? Environ. Toxicol. Chem. 23, 1344–1354.

Flaherty, C.M., Dodson, S.I., 2005. Effect of pharmaceuticals on *Daphnia* survival, growth and reproduction. Chemosphere 61, 200–207.

Froehner, K., Backhaus, T., Grimme, L.H., 2000. Bioassays with *Vibrio fischeri* for the assessment of delayed toxicity. Chemosphere 40, 821–828.

Guillard, R.L., Lorenzen, C.J., 1972. Yellow-green algae with chlorophyllide *c*. J. Phycol. 8, 10–14.

Halling-Sørensen, B., 2000. Algal toxicity of antibacterial agents used in intensive farming. Chemosphere 40, 731–739.

Halling-Sørensen, B., Holten-Lützhøft, H.C., Andersen, H.R., Ingerslev, F., 2000. Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. J. Antimicrob. Chemother. 46 (Suppl. S1), 53–58.

Hartmann, A., Alder, A.C., Koller, T., Widmar, R.M., 1998. Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater. Environ. Toxicol. Chem. 17, 377–382.

Hendriks, A.J., Maas-Diepeveen, J.L., Noordsij, A., Van der Gaag, M.A., 1994. Monitoring response of XAD-concentrated water in the Rhine delta: a major part of the toxic compounds remains unidentified. Water Res. 28, 581–598.

Hilton, M.J., Thomas, K.V., 2003. Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography–electrospray tandem mass spectrometry. J. Chromatogr., A 1015, 129–141.

Hirsch, R., Ternes, T., Haberer, K., Kratz, K.L., 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225, 109–118.

Holten-Lützhøft, H.C., Halling-Sørensen, B., Jørgensen, S.E., 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. Arch. Environ. Contam. Toxicol. 36, 1–6.

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Sci. Total Environ. 346, 87–98.

Kim, S., Aga, D.S., 2007. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. J. Toxicol. Environ. Health, Part B 10, 559–573.

Kümmerer, K., 2009. Antibiotics in the environment – a review – Part II. Chemosphere 75, 435–441.

Lanzky, P.F., Halling-Sørensen, B., 1997. The toxic effect of the antibiotic metronidazol on aquatic organisms. Chemosphere 35, 2553–2561.

Maul, J.D., Schuler, L.J., Belden, J.B., Whiles, M.R., Lydy, M.J., 2006. Effects of the antibiotic ciprofloxacin on stream microbial communities and detritivorous macroinvertebrates. Environ. Toxicol. Chem. 25, 1598–1606.

Näslund, J., Hedman, J.E., Agestrand, C., 2008. Effects of the antibiotic ciprofloxacin on the bacterial community structure and degradation of pyrene in marine sediment. Aquat. Toxicol. 90, 223–227.

NEN (Nederlands Normalisatie Instituut), 1980. NPR 6503. Water-Necessaries, Method and Medium for the Culture of *Daphnia magna* and the Cultivation of the Algae Required as Food. NEN, Delft, The Netherlands.

Pikkemaat, M.G., Oostra-van Dijk, S., Schouten, J., Rapallini, M., van Egmond, H.J., 2008. A new microbial screening method for the detection of antimicrobial residues in slaughter animals: the Nouws antibiotic test (NAT-screening). Food Control 19, 781–789.

Rang, H.P., Dale, M.M., Ritter, J.M., 1995. Pharmacology, third ed. Churchill Livingstone, New York.

Roberts, P.H., Thomas, K.V., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Sci. Total Environ. 356, 143–153.

Robinson, A.A., Belden, J.B., Lydy, M.J., 2005. Toxicity of fluoroquinolone antibiotics to aquatic organisms. Environ. Toxicol. Chem. 24, 423–430.

Schrap, S.M., Rijs, G.B.J., Beek, M.A., Maaskant, J.F.N., Staeb, J., Stroomberg, G., Tiesnitsch, J., 2003. Humane en veterinaire geneesmiddelen in Nederlands oppervlaktewater en afvalwater. RIZA Rapport 2003.023.

Sweetman, S.C., 2002. Martindale the Complete Drug Reference, 33rd ed. Pharmaceutical Press, London, UK.

Thomulka, K.W., McGee, D.J., Lange, J.H., 1993. Detection of biohazardous materials in water by measuring bioluminescence reduction with the marine organism *Vibrio harveyi*. J. Environ. Sci. Health, Part A 28, 2153–2166.

Van Kooten, O., Snel, J.F.H., 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynth. Res. 25, 147–150.

Vieno, N.M., Tuhkanen, T., Kronberg, L., 2006. Analysis of neutral and basic pharmaceuticals in sewage treatment plants and in recipient rivers using solid phase extraction and liquid chromatography–tandem mass spectrometry detection. J. Chromatogr., A 1134, 101–111.

Yang, G.L.-H., Ying, G.-G., Su, H.-C., Stauber, J.L., Adams, M.S., Binet, M.T., 2008. Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga *Pseudokirchneriella subcapitata*. Environ. Toxicol. Chem. 27, 1201–1208.

Ye, Z., Weinberg, H.S., Meyer, M.T., 2007. Trace analysis of trimethoprim and sulphonamide, macrolide, quinolone, and tetracycline antibiotics in chlorinated drinking water using liquid chromatography electrospray tandem mass spectrometry. Anal. Chem. 79, 1135–1144.

Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. Lancet 335, 1789–1790.