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Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae

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Abstract

The influence of antimicrobial agents approved as veterinary drugs in Japan on the growth of green algae, *Selenastrum capricornutum* and *Chlorella vulgaris*, was studied in accordance with the OECD guidelines for testing chemicals. Among the agents tested, growth inhibitory activity was very varied, i.e. erythromycin showed the strongest activity (EC_{50}, 50% effective concentration, =0.037 mg/l), sulfa drugs had activity to some extent (EC_{50}s of sulfamethoxazole, sulfadiazine, and sulfadimethoxine were 1.5, 2.2, and 2.3 mg/l, respectively), but ampicillin and cefazolin did not inhibit growth (EC_{50}s > 1000 mg/l). We also investigated synergistic effect of combining sulfa drugs with trimethoprim or pyrimethamine, which are commonly used as a combined drug. By adding trimethoprim, the growth inhibitory activity of sulfamethoxazole and sulfadiazine was significantly enhanced. Growth inhibition by sulfa drugs was reduced by the addition of folic acid, indicating that they inhibit folate synthesis in green algae.

Keywords: Algal toxicity; Combined drug; Synergistic effect; Folate

1. Introduction

Antimicrobial agents are widely used and indispensable not only for human medication, but also in the veterinary field for livestock and companion animals. Some antimicrobial agents administered to animals are metabolized, excreted and thereby released into the environment. It has been reported that several antimicrobial agents are detected in the environment because they are poorly metabolized in the body and/or degraded at ordinary sewage disposal plants (Halling-Sørensen et al., 1998; Al-Ahmad et al., 1999). Such antimicrobial agents can have a deleterious effect on sensitive microorganisms in the field, which may cause a disorder of the ecosystem. Therefore, the potential impact of antimicrobial agents on the ecosystem must be evaluated as closely as other hazardous chemicals. Since

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photoautotrophic microalgae are the primary producers of essential nutrients in the ecosystem, toxicity against these organisms is considered to be of particular importance.

Environmental toxicity tests defined by the OECD guidelines for testing chemicals are widely accepted and performed and the "Alga, growth inhibition test" (No. 201) is frequently used. For example, Lüzhøft et al. (1999) and Halling-Sørensen (2000) have reported that several antimicrobial agents used in Europe have toxic effects on algae.

In most cases, these toxicity tests are performed using individual chemicals. However, two antimicrobial agents are sometimes administered to animals simultaneously as a combined drug. Therefore, it is likely that organisms will be exposed to plural antimicrobial agents in the ecosystem. For example, ST (a combination of a sulfur drug and trimethoprim) or a similar combined drug (pyrimethamine is often used as a substitute for trimethoprim) is widely used in veterinary medicine. It is expected that combined drugs would exhibit synergistic influence beyond their individual influences.

In this study, growth inhibitory tests of several antimicrobial agents approved as veterinary drugs in Japan were carried out using algae in accordance with the OECD guidelines. In addition, we investigated the synergetic effect of combined drugs consisting of sulfa drugs and trimethoprim or pyrimethamine in the growth inhibition test.

2. Materials and methods

2.1. Algal species

Selenastrum capricornutum (ATCC22662) and *Chlorella vulgaris* (ATCC16487) were obtained from the American Type Culture Collection (ATCC). Both strains were maintained in recommended media. Before the test, they were cultivated for at least 6 days in OECD medium following the OECD-guideline No. 201.

2.2. Chemicals

Sulfadimethoxine (SDM, sodium salt), trimethoprim (TMP), pyrimethamine (PMT) were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfamethoxazole (SMZ), oxytetracycline (OTC, hydrochloride salt), cefazolin sodium salt (CFZ, sodium salt), ampicillin (ABPC, sodium salt), thiamphenicol (TP), erythromycin (EM), tylosin tartate (TST), and norfloxacin (NFLX) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sulfadiazine (SDA), dihydrostreptomycin (DSM) were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). N_4-acethyl sulfa drugs (AcSAs) derived from each sulfa drugs (SAs) were synthesized as described below (Section 2.4). Other chemicals (reagent grade) were purchased from Wako Pure Chemical Industries, Ltd., Sigma Chemical Co., Kanto Chemicals Co., Inc. (Tokyo, Japan), ICN Pharmaceuticals, Inc. and Nacalai Tesque (Kyoto, Japan).

2.3. Procedure for the growth inhibitory test

Growth inhibitory tests were performed following the OECD guideline for testing chemicals No. 201 with minor modifications. In brief, a conical flask (200 ml) containing 100 ml of distilled water was plugged with a silicone-foam plug and autoclaved at 121 °C for 20 min. Condensed medium solution sterilized by filtration (pore size: 0.2 μm) was added and precultivated algae cells were inoculated. The culture was maintained at 25 °C, at 70% relative humidity, and illuminated at 5000 lx (about 11 W/m^2) from white fluorescent lamps in a growth chamber (model ALWG modulated, Hirasawa Works, Tokyo, Japan). The flasks were shaken and changed in position relative to each other 4 times per day to equalize irradiating light intensity. Cell growth was measured at 1 day intervals by an automatic hematology analyzer (Celltac MEK-5133, Nihon Kohden Co., Tokyo, Japan).

At first, "range-finding test", which consisted of three concentrations of test substance in geometric series (common ratio = 10) and negative control, was carried out to determine the approximate EC_{50}. Secondary, the main test was carried out, in which negative control and five concentrations of test substance in geometric series (common ratio = 2) determined so that the above EC_{50} value located in the center of the concentration's distribution by the consequence of range-finding test was used. Each concentrations and negative control of both tests were designed triplicate.

2.4. Synthesis of N_4-acethyl sulfa drugs

SAs were dissolved in 0.05 mol/l sulfuric acid and acetone (1/1), and chemical equivalent plus more of acetic anhydride was added in drops with stirring. Occasionally, products and source materials were analyzed by thin layer chromatography [developing solvent: chloroform and methanol (20/1), plate: Silica Gel WF_{254}s/aluminium plate (Merck, Darmstadt, Germany)]. After the spot of source material disappeared under UV irradiation, the reaction solution was neutralized by 1 mol/l of sodium hydroxide and concentrated to half the original volume under reduced pressure. The precipitated crystals were separated by filtration. The crystals were recrystallized in methanol twice, and dried at 105 °C under reduced pressure. The structure of the product was confirmed by determining the nuclear magnetic resonance spectrum (^{1}H, 270 MHz).

2.5. Procedure for the inhibitory test of combined substances and folic acid

The procedure for the growth inhibitory test of ST combined substances was almost identical to that for single substances. The excretion ratio of the test substances was estimated from their excretion rate after intravenous administration to pigs according to Shimoda et al. (1981, 1990, 1997), and by Nouws et al. (1991). The ratio was SMZ:AcSMZ:TMP = 20:105:3, SDM:AcSDM:PMT = 167:8:1 and SDA:AcSDA:TMP = 42:24:1. Folic acid was added at concentrations of 20 ng/l and 100 ng/l.

2.6. Statistical methods

The Eco-Tox R1.1 program (composed by Yoshioka et al.), distributed by the Japan Society of Environmental Toxicology, was used to analyze data. The area under the growth curve at 72 h was analyzed by the logit (planimetry) method. Significance differences between groups were determined using the Student's *T*-test for unpaired samples.

2.7. HPLC conditions

OTC determination was performed using a Chemcosorb 3C8 column (4.6 mm i.d. × 125 mm L, Chemco Scientific Co., Ltd., Osaka, Japan) and other determinations using Capcell Pak C18 columns (UG120 S-5 μm 4.6 mm i.d. × 250 mm L, Shiseido Co., Ltd., Tokyo, Japan). Column temperature was 40 °C. Mobile phase was 0.01 mol/l oxalic acid (pH = 3.0), methanol and acetonitrile (7/1/1.5) for OTC, 0.01 mol/l KH_2PO_4 and acetonitrile (88/12) for ABPC and CFZ, water and acetonitrile (85/15) for TP, water, methanol and acetic acid (60/40/0.5) for SAs and AcSAs, 0.3% (v/v) of H_3PO_4 (adjusted to pH = 3.0 by triethylamine) and acetonotrile (85/15) for NFLX. The flow rate was 1.0 ml/min. All detections were performed by UV absorption, in which the wavelength was 350 nm for OTC, 210 nm for ABPC and CFZ, 240 nm for TP, 270 nm for SAs and AcSAs, and 278 nm for NFLX.

3. Results and discussion

The growth inhibition test for individual antimicrobial agents was carried out using freshwater green algae, *S. capricornutum* and *C. vulgaris*, which are used as standard strains in the OECD test (Table 1). Our results indicate that *S. capricornutum* was more sensitive in general to antimicrobial agents than *C. vulgaris*. Kasai and Hatakeyama (1993) and Kasai et al. (1993) also found the same tendency in the sensitivity of these algae to several herbicides. EM showed the strongest growth inhibition (EC_{50} = 0.037 mg/l) against *S. capricornutum*, followed by DSM (0.11 mg/l), OTC (0.34 mg/l) and TST (0.41 mg/l). SAs also exhibited growth inhibition to some extent (EC_{50} = 1.53–2.30 mg/l) amongst the agents tested. In contrast, CFZ and ABPC had no influence on the growth (EC_{50} > 1000 mg/l). In other reports, several herbicides have shown similar toxicities to

Table 1
The results of the growth inhibition test against green algae using antimicrobial agents approved for veterinary use in Japan

Substance	*Selenastrum capricornutum*			*Chlorella vulgaris*		
	EC_{50} (mg/l)	95% confidence interval (mg/l)	NOEC (mg/l)	EC_{50} (mg/l)	95% confidence interval (mg/l)	NOEC (mg/l)
Sulfadimethoxine (SDM)	2.30	2.09–2.54	0.529	11.2	7.37–15.0	<20.3
Sulfamethoxazole (SMZ)	1.53	1.40–1.68	0.614	–	–	–
Sulfadiazine (SDA)	2.19	1.95–2.42	<1.00	–	–	–
Oxytetracycline (OTC)	0.342	0.321–0.364	0.183	7.05	6.50–7.55	<3.58
Cefazolin Na (CFZ)	>1000	– –	>1000	–	–	–
Ampicillin (ABPC)	>1000	– –	>1000	>1000	– –	>1000
Dihydrostreptomycin (DSM)	0.107	0.0933–0.121	<0.039	–	–	–
Thiamphenicol (TP)	8.86	8.27–9.50	4.06	522	489–580	198
Erythromycin (EM)	0.0366	0.0358–0.0399	0.0103	33.8	31.3–36.4	12.5
Tylosin tartate (TST)	0.411	0.387–0.437	0.206	–	–	–
Norfloxacin (NFLX)	16.6	15.5–17.8	4.01	10.4	9.72–11.0	4.02
Trimethoprim (TMP)	80.3	74.4–86.7	25.5	–	–	–
Pyrimethamine (PMT)	5.06	4.66–5.50	0.752	–	–	–
Pentachlorophenol sodium salt[a]	0.160	0.150–0.170	0.105	8.40	7.84–8.97	5.02

–: not tested.
– –: above the limit of detection.
EC_{50}: 50% effective concentration, the concentration of a substance that will reduce cell growth to 50% of the negative control.
NOEC: maximum no-effect concentration.
[a] Positive control.

S. capricornutum and *C. vulgaris* as the compounds tested in this study. For example, the EC_{50}s of symetryn, pretilachlor, and thiobencarb for *S. capricornutum* were 10, 2.5, and 38.6 μg/l, respectively (Kasai et al., 1993). Based on the mechanism of action of most herbicides, it is possible that they will also inhibit the growth of green algae in the environment. Therefore, the anitimicrobial agents showing growth inhibitory activity in this study, particularly EM, would be expected to cause damage to green algae in the ecosystem if they were released into the field at concentrations similar to their EC_{50} values. EM inhibits the growth of prokaryotes by binding to the 50S ribosome, thereby inhibiting transpeptidation. However, this mechanism of action will not inhibit the growth of eukaryotes, so the inhibition of the growth of green algae by EM cannot be satisfactorily explained by the known mechanisms of action of this compound.

Recently, the use of SAs in human medicine has been reduced because more effective antibiotics are available. However, SAs are still widely used in veterinary medicine and the livestock industry because of their low cost. The results presented in this study indicate that excessive use of SAs in livestock may pose a risk to green algae in the environment. However, this risk may be reduced as SAs are metabolized in animals to AcSAs, which were found to have a much weaker growth inhibitory effect than that of the parent SAs (Table 3).

The concentrations of the antimicrobial agents in the culture medium during incubation with *S. capricornutum* were measured. As shown in Fig. 1, a decrease in OTC, TST, ABPC and CFZ was observed, although other agents did not decrease significantly. In particular, OTC decreased most rapidly and was not detectable within the media within 2 days. Asker and Habib (1991) reported that tetracycline and its analogues, including OTC, are easily oxidized in the presence of oxygen and light, conditions that accord with those of the culture medium of the algae used in this study.

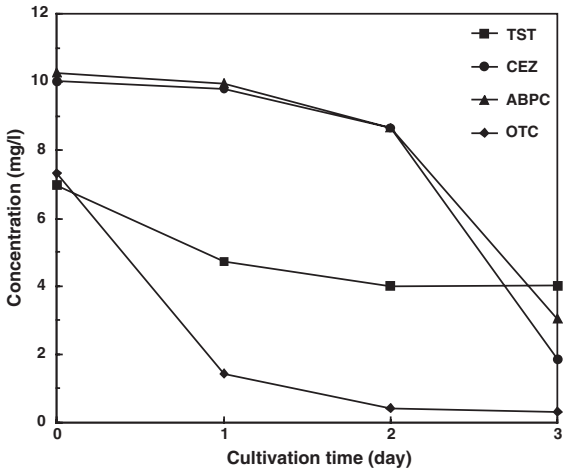

Fig. 1. Decrease in concentration of test agents in the growth inhibition test. Other test substances did not show a significant decrease during the course of the experiment.

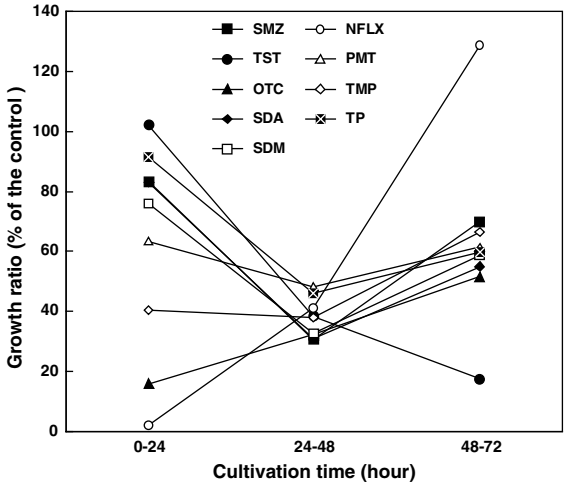

Fig. 2. Variation of the growth rate. Each test substance was used at the EC_{50} concentration. Growth rate was determined as increase in cell number/h.

Fig. 2 shows the change in the growth rate of *S. capricornutum* in the presence of the test agents. Most agents gave a "V-shaped" curve, indicating that the growth was inhibited strongly during the middle of the incubation period (1–2 days). In contrast, OTC and NFLX inhibited growth early during the incubation period (0–1 day), allowing growth of the algae to subsequently recover. Exceptionally, TST strongly inhibited growth during the late incubation period (2–3 days).

OTC showed strong growth inhibition for *S. capricornutum* and *C. vulgaris* despite of its rapid disappearance. OTC inhibited specifically early in the incubation period, suggesting that growth inhibition by OTC occurred when its concentration was at a relatively higher level. Therefore, it is more likely that OTC will cause damage to algae when it is released continuously into the environment.

To investigate the synergistic influence of combined drugs on the growth of green algae, SAs and TMP or PMT (TMPs) were simultaneously added to *S. capricornutum* culture. In this experiment, the concentration of TMPs was fixed at the no observed effect concentration (NOEC) and the concentration of the SAs were altered. These combined drugs are frequently used in the veterinary field in Japan. Combination of SMZ and SDA with TMP rendered the growth inhibitory activity significantly increased in comparison with their individual activities (Table 2, Fig. 3A–C). On the other hand, combination of SDM with PYR did not show such an effect.

Table 2
Synergistic growth inhibition between SA and TMP or PMT

Substances	EC_{50} (mg/l)	95% confidence interval (mg/l)	NOEC (mg/l)
SMZ	1.53	1.40–1.68	0.614
SDA	2.19	1.95–2.42	<1.00
SDM	2.30	2.09–2.54	0.529
TMP	80.3	74.4–86.7	25.5
PMT	5.06	4.66–5.50	0.752
SMZ + TMP[a]	0.275[b]	0.239–0.309[b]	0.2[b]
SDA + TMP[a]	0.465[b]	0.419–0.517[b]	<0.125[b]
SDM + PMT[a]	2.36[b]	2.17–2.57[b]	1.00[b]

TMP and PMT were used at their minimum no-effect observed concentrations (NOEC) and the concentration of SA was varied.

[a] TMP and PMT were used at their NOECs.

[b] Concentration of SA in each combination.

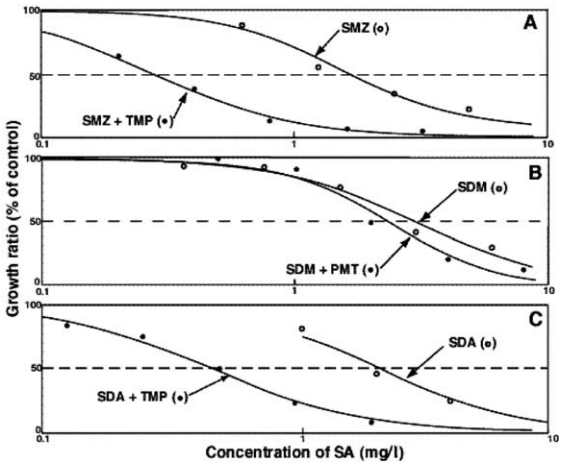

Fig. 3. The dose–response curve of sulfur drugs combined with TMP or PMT (A: SMZ, B: SDM, C: SDA).

SAs are thought to be partly metabolized to AcSAs in the bodies of animals (Vree et al., 1985). Therefore, the test of combined drugs was done by using the combinations above mixed with the corresponding AcSAs at a ratio according to the concentrations detected in the urine of pigs fed with SAs. A similar synergistic effect to that described above (Table 2 and Fig. 3) was observed with combinations of SMZ, TMP, and AcSMZ (Table 3). On the other hand, combination of SDM or SDA with their acetylate and PMT or TMP did not show a synergistic effect on growth in excretion ratio. A reason must be that the concentration of TMP used was not enough to express synergistic influence in combination with SDM or SDA. These results indicate that several combined drugs that show a synergistic effect in vitro may have an actual synergistic effect on algae in ecosystem although excretion ratio can vary in animal condition or other factors. The synergistic effect observed by the combination of SAs and TMPs in this study indicates that the simultaneous release of several antimicrobial agents may result in greater toxicity to microorganisms in the environment than the release of the same agents individually.

The growth inhibitory activity of the combination of SDA and TMP was significantly reduced by the addition of 20 ng/l of folic acid to the medium. Significantly, folic acid exhibited a similar effect when SDA was tested alone, but not when TMP was tested alone (Fig. 4). Both SAs and TMPs inhibit the folate synthesis pathway in bacteria, but their inhibition sites are different. SAs inhibit dihydropterinic acid synthetase (DHPS), thereby inhibiting the synthesis of folic acid. On the other hand, TMPs inhibits dihydrofolic acid reductase (DHFR), which converts folic acid to 7,8-dihydrofolic acid (7,8-DHF) and 5,6,7,8-tetrahydrofolic acid (5,6,7,8-THF), both active forms of folic acid suitable for utilization. Therefore, the synergistic effect of the combination of SAs and TMPs is likely to be due to the cumulative effect of their actions on two different sites in the folate biosynthesis pathway. Since SAs block the synthesis of folate, the growth inhibitory effect of this compound can be reversed by the addition of folate. In contrast, TMP blocks enzymes downstream of folate in the synthesis pathway, thus addition of folate will not reverse the growth-inhibiting effect of this compound. Since algae

Table 3
Synergistic growth inhibition by the ST combined drug estimated to be excreted by pigs

Substance and mixing ratio (SA:AcSA:TMPs)	EC_{50} (mg/l)	95% confidence interval (mg/l)	NOEC (mg/l)
SMZ:AcSMZ:TMP (20:105:3)	0.784[a]	0.717–0.856[a]	0.2[a]
SDM:AcSDM:PMT (167:8:1)	2.17[a]	1.95–2.40[a]	0.5[a]
SDA:AcSDA:TMP (42:24:1)	2.08[a]	1.88–2.33[a]	0.5[a]
AcSMZ	>100	–	80
AcSDM	>100	–	>100
AcSDA	>100	–	>100

–: above the limit of detection.

[a] Concentration of SA in each combination.

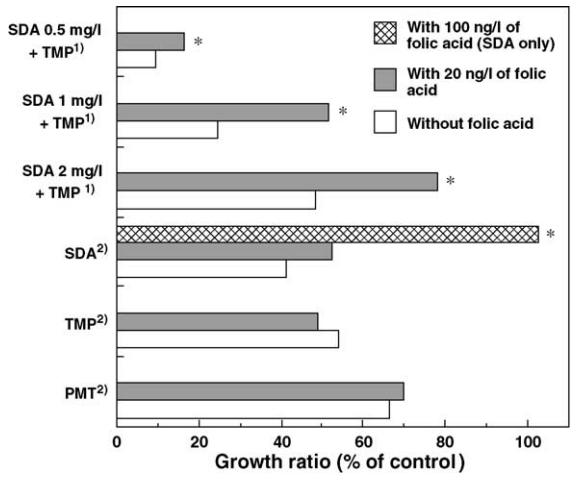

Fig. 4. Recovery of growth inhibition by the addition of folic acid. *Observed significant difference to negative control (without FA) ($P < 0.05$). 1)Concentration of SDA in the combination, TMP was used at the NOEC. 2)Used at the EC_{50} concentration.

also have a similar folate synthesis pathway (Pratt and Johnson, 1965), the growth inhibitory effect of SAs on these organisms is likely to be the result of the same inhibitory mechanism. Therefore, algal cells could survive in the presence of SAs, but not TMP, when folic acid was added to the medium.

4. Conclusion

In this study, we evaluated the influence of antimicrobial agents frequently used as veterinary drugs in Japan on the growth of green algae following OECD guidelines. Antimicrobial agents would be expected to principally influence the growth of prokaryotes, such as bacteria and cyanobacteria, but not affect the growth of eukaryotes, such as green algae. Contrary to this expectation, several agents showed strong growth inhibitory activity to green algae. Furthermore, this activity was synergistically enhanced for combined drugs that are frequently used in the veterinary field. As green algae are located on the lowest level of the water-ecosystem pyramid, it is of great concern that the effect on algal flora from agents released into the environment will extend to the whole ecosystem. It is also likely that agents showing toxic activity to algae will exert similar effects on other eukaryotic organisms in soil and water, such as insects and zooplankton. Therefore, a detailed cohort study of the fate of antimicrobial agents administered to animals for veterinary use should be conducted to determine their potential influence on the ecosystem.

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