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Effects of a Realistic Mixture of Antibiotics on Resistant and Nonresistant Sewage Sludge Bacteria in Laboratory-Scale Treatment Plants

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Abstract The detection of antibiotics and resistant bacteria in sewage treatment plants (STPs) has stimulated a discussion on the origin and selection of resistant bacteria during sewage treatment. Currently, there is little data available regarding the effects of realistic mixtures of antibiotics on the bacteria present in the aeration tanks of STPs. In this study we used four laboratory-scale sewage treatment plants (LSSTPs) to study the effects of antibiotics on bacteria during sewage treatment under standardized conditions. Two plants were fed with a mixture of antibiotics at two concentration levels based on the average annual input of antibiotics into German municipal STPs. The total operational period was 84 days. A multiresistant bacterium (*Acinetobacter baumannii*) was added twice to two of the plants. The fate of the multiresistant bacterium was monitored. The mix of antibiotics did not affect the purification efficiency. The presence of the antibiotics did not favour the multiresistant bacterium. No difference was detected between the test plant and the controls.

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Most of the pharmaceuticals used in medicine are only partially metabolized by patients, with the remainder being discharged into municipal waste water along with excreta. Antibiotics are among the most intensively used and important groups of pharmaceuticals. Wise (2002) estimated total antibiotic market consumption worldwide to lie between 100,000 and 200,000 t. According to data supplied by the European Federation of Animal Health (FEDESA 2001), 13,288 t of antibiotics were used in the European Union and Switzerland, of which 65% was used in human medicine. The β-lactams (penicillins, cephalosporins, and carbapenems) account for approximately two-thirds of the total volume in most countries, according to available data. On average, 70% of the compounds are excreted unchanged.

Laboratory testing has shown that most antibiotics, including β-lactams, sulfonamides and quinolones, are either not biodegradable at all or are only biodegradable to a minor extent in aquatic environments (Al-Ahmad et al. 1999; Alexy et al. 2004). Accordingly, antibiotics have been detected in the $\mu g\ l^{-1}$ range in municipal sewage, in the effluent of sewage treatment plants (STPs), in surface water, and in groundwater (Benito-Pena et al. 2006; Kümmerer 2008). The selection and development of bacteria that are resistant to antibiotics is one of the biggest concerns with regard to the use of antimicrobials. Resistant

and even multiresistant pathogenic bacteria have been detected in wastewater and STPs, as well as in other environmental compartments (Kümmerer 2004). Very little data is available to date describing elimination rates and the effects of antibiotics on bacteria present in the aeration tanks of STPs (De Gelder et al. 2005; Reinthaler et al. 2003).

Viable plate count and culture-dependent techniques select for certain bacteria and may recover only 1–15% of the bacterial populations in activated sludge and other environmental samples, leading to a false picture of the true bacterial population structure (Amann et al. 1995; Wagner et al. 1993). Because of its simplicity and high reproducibility, the impact of quinone profile analysis on the analysis of bacterial populations in activated sludge and wastewater treatment systems has been shown by many authors (Hu et al. 2001; Lim and Ahn 2004; Songprasert et al. 2004); for example, quinone profiling studies have indicated that the number of *Acinetobacter* cells is low in the standard activated sludge process and the enhanced biological phosphate removal process (Hiraishi 1989). However, *Acinetobacter* species were isolated from different activated sludge plants (Wiedmann-Al-Ahmad et al. 1994). Other important chemotaxonomic markers in bacterial populations are the polyamines (Auling 1992; Busse and Auling 1988). The combination of quinone and polyamine analysis allows a better comparison of complex bacterial matrices, i.e., activated sludge.

In this study, we applied laboratory-scale sewage treatment plants (LSSTPs) to study the effects of antibiotics on resistant and nonresistant bacteria in biological sewage treatment. Quinone and polyamine profiling was used to study the fate of a multiresistant *Acinetobacter baumannii* strain in the presence and absence of a realistic mix of antibiotics in these LSSTPs. Since *A. baumannii* is a bacterium present in the environment, it serves as an example for antibiotic resistance in the environment.

Methods and Materials

Laboratory-Scale Sewage Treatment Plants (LSSTPs)

Four LSSTPs were operated in parallel according to the Organization for Economic Cooperation and Development (OECD) 303A test guidelines (OECD 2001). The volume of the aeration vessel was 3.5 l and that of the sedimentation tank was 1.5 l. The wasting rate of 200 ml sludge was taken to keep a dry matter content of 2.5 g l^{-1} in the aeration tank. The activated sludge flow rate in the sedimentation tank amounted to 0.5 l h^{-1}. The sedimented sludge was recycled for 15 s every 15 min using a pump device that was air-based. The sludge age was calculated to be 31 days, which is typical for LSSTPs (Metzner 1992). The plants were inoculated with sewage sludge (dry matter content 4.2 g l^{-1}) from a municipal sewage treatment plant (STP; Abwasserzweckverband Breisgauer Bucht, Forchheim, Germany; 650,000 inhabitant equivalents). Prior to use, the sludge was washed three times and aerated for 24 h. The sludge from the LSSTPs was not mixed to allow for the monitoring of population dynamics by chemotaxonomy using biomarkers. The composition of the synthetic sewage stock solution in mg l^{-1} was as follows: 27 calcium chloride dehydrate, 1,067 casein peptone, 733 meat extract, 200 ammonium chloride, 13 magnesium sulfate heptahydrate, 47 sodium chloride, 1,117 potassium hydrogen phosphate, and 1,567 sodium hydrogen carbonate. All components were purchased from Merck (Darmstadt, Germany). The synthetic sewage was fed from a concentrate which was kept at 4°C and added continuously at a load of 74 mg l^{-1} dissolved organic carbon (DOC). The hydraulic residence time was 6 h. The resulting sludge loading was 0.1−0.25 mg (mg d)$^{-1}$ in the aeration vessel. DOC elimination was calculated according to the test guidelines (OECD 2001). Analyses were performed as described in the following section. The total test period was 84 days. The experimental setting is depicted in Table 1. After a 3-week acclimatization period, two test plants (referred to as "test" and "control 2") were additionally fed with a mixture of antibiotics. In its composition, the mixture of the applied antibiotics reflected the annual average input of antibiotics into German municipal STPs (Kümmerer and Henninger 2003). The applied concentrations of the antibiotics are summarized in Table 2. The amounts of antibiotics used were 10-to 100-fold that expected for the average annual concentrations of antibiotics in municipal sewage influents in Germany, calculated using data on the annual levels of antibiotic and water consumption (Kümmerer and Henninger 2003). The multiresistant *Acinetobacter baumannii* was added in high concentration to the test plants on days 27 and 55 [9.2×10^8 colony-forming units (CFU) per liter and 5.1×10^8 CFU per liter] in order to study its fate under antibiotic pressure. The *Acinetobacter* was isolated in the Institute for Environmental Medicine and Hospital Epidemiology, University Hospital of Freiburg from a patient

Table 1 Test outline

	Blank	Control 1	Control 2	Test
Synthetic sewage inoculum from municipal sewage treatment plant	+	+	+	+
Antibiotic mix			+	+
Multiresistant *Acinetobacter baumannii*		+		+

Table 2 Composition of the antibiotic mixture (concentrations are 10- to 100-fold the calculated annual average input of antibiotics in municipal sewage influents in Germany)

Compound	Concentrations applied in the laboratory-scale sludge treatment plants [μg l^{-1}]	
	Low (days 27–55)	High (days 55–84)
Amoxicillin	25.7	257
Penicillin V	7.5	75.9
Cefazolin	4.5	45
Cefuroxime	4.5	45
Ceftazidime	4.5	45
Doxycycline	0.65	6.5
Erythromycin	1.7	17
Vancomycin	0.36	3.6
Ciprofloxacin	1.6	16
Sulfamethoxazole[a]	133.5	1335
Trimethoprim[a]	26.7	267

[a] Trimethoprim is not an antibiotic applied in human medicine as a single substance. It is applied in combination with sulfamethoxazole only (5:1)

(ID: ACI-142) and shows the resistance pattern given in Table 3. The fate of the multiresistant bacterium was monitored by using chemotaxonomy [high performance liquid chromatography (HPLC)] and classical microbiological means (see below).

Oxygen concentration, pH, and temperature were measured on the same days as the sampling for chemotaxonomy and for monitoring of resistance took place. DOC elimination was monitored at least three times a week during the course of the test. Three replicates were analyzed. The samples were centrifuged (Minifuge 2; Heraeus, Hanau, Germany) and filtered (cellulose nitrate, 0.45 μm; Sartorius, Göttingen, Germany) prior to measurement. DOC samples were stored at 4°C until analysis. DOC was determined according to the European standard procedure DIN EN 1484 (1997) with a total organic carbon (TOC) analyzer (TOC 5000; Shimadzu GmbH, Duisburg, Germany). Sludge volume and dry matter content were also determined according to German standard methods for the examination of water, waste water and sludge (DIN 38 412 1996).

Microbiological Monitoring of Bacteria in the Aeration Tanks

The agar dilution procedure was used to monitor multiresistant bacteria in the laboratory-scale sewage treatment plants (LSSTPs). The liquid agar (Kappesser 1990) was cooled to 48°C and then mixed with the antibiotic solution to simulate the situation in STPs and select for multiresistant bacteria (Table 4, concentration refers to the minimum inhibition concentration of each antibiotic against sensitive pathogenic bacteria). Temperature was always kept below 50°C to avoid thermal decomposition of the antibiotics. The liquid agar was allowed to cool to room

Table 3 Resistance characteristic of the isolated *Acinetobacter baumannii* (ID: ACI-142, source: Department of Environmental Health Sciences, University Medical Centre Freiburg)

Antibiotic	Minimal inhibitory concentration (MIC) [mg l^{-1}]	Systemic*
Tobramycin	>8	Resistant
Piperacillin	>64	Resistant
Cefotaxime	>32	Resistant
Ceftazidime	>16	Resistant
Cefuroxime	>16	Resistant
Imipenem	≤4	Susceptible
Meropenem	≤4	Susceptible
Amoxicillin/potassium clavulanate	>16/8	Resistant
Ciprofloxacin	>2	Resistant
Trimethoprim/sulfamethoxazole	>2/38	Resistant
Cefepime	>16	Resistant
Cefpodoxime	>2	
Gentamicin	>8	Resistant
Amikacin	32	Intermediate
Nitrofurantoin	>64	
Norfloxacin	>8	
Aztreonam	>16	Resistant
Ticarcillin/potassium clavulanate	16	Susceptible
Levofloxacin	>4	Resistant
Ampicillin	>16	

* Interpretations based on National Committee for Clinical Laboratory Standards (NCCLS) M100-S7 Jan. 97

Table 4 Antibiotic concentrations used in agar for the isolation of multiresistant bacteria from the laboratory-scale sludge treatment plants

Antibiotic	Concentration [mg l^{-1}]
Amoxicillin	32
Cefuroxime	32
Imipenem	16
Gentamicin	16
Erythromycin	8
Doxycycline	16
Ciprofloxacin	8
Sulfamethoxazole	76
Trimethoprim	4
Vancomycin	32

temperature and was then stored at 4°C until use. The resulting agar plates were used within 24 h. Colony-forming units (CFUs) were determined once a week. Medium without antibiotic mix was used in parallel as a negative control (no selection effect).

Polyamines, ubiquinones, and menaquinones were analyzed by HPLC as biomarkers to monitor bacterial population dynamics. Sampling was performed on the same days as for the DOC. Samples were taken 24 times during the course of the trial. All the chemicals used for extraction, clean up, and analysis of these compounds were of at least 98.5% purity, whereas the solvents used for the HPLC analysis were of gradient grade (LiChrosolv®; Merck, Darmstadt, Germany).

Biomarker extraction and analysis were described elsewhere in detail (Collins and Jones 1981; Hamana and Matsuzaki 1992; Hiraishi 1988; Scherer and Kneifel 1983). Analysis was done by HPLC and ultraviolet/visual (UV/Vis) detection. HPLC apparatus (Shimadzu, Duisburg, Germany) consisting of the software package Class LC10, the communication interface CBM-10A, and two LC-10 AT VP-pumps was used. Flow was set at 1 ml min^{-1} with methanol/isopropyl ether (7:2, v/v) isocratic elution. The column was a Nucleosil 100-5 C18, 250 × 4 mm (Macherey-Nagel, Düren, Germany). A SIL-10 autosampler with CTO-10 AS VP column oven (temperature set at 25°C) was used. The UV/Vis detector (SPD-10 A) was set to 275 nm for detection of ubiquinones, and to 270 nm for detection of menaquinones. Better identification of menaquinones using a diode array detector (SPD-M10) was conducted when necessary.

For the polyamines a binary gradient was applied beginning with 40% acetonitrile/60% water and increased to 85% acetonitrile/15% water. Polyamines were detected with a fluorescence detector (RF-10A) after derivatization with dansyl chloride. External standards were putrescine, diaminopropane, cadaverine, spermidine, and spermine (Sigma, Deisenhofen, Germany). Hydroxyputrescine and sym-homospermidine were isolated from bacteria: sym-homospermidine from S*phingomonas paucimobilis* (DSM 1098), and hydroxyputrescine from *Burkholderia cepacia* (DSM 50180). For the quantification of ubiquinones external standards were used (Q 6, Q 7, Q 9, Q 10; Sigma, Deisenhofen, Germany). For the menaquinones vitamin K (Sigma, Deisenhofen, Germany) and other components were used as standards. The other menaquinones were not commercially available; therefore, they had to be isolated from suitable Gram-positive bacteria. To identify the extracted menaquinone mixture by HPLC, main and secondary components of menaquinones were extracted from different Gram-positive bacteria which were cultivated in different growth media according to culture media described by the German Type Culture collection (DSMZ, Braunschweig, Germany). All of the menaquinone components which were extracted from bacteria and then HPLC-identified are shown in Table 5.

Statistical Analysis

One way analysis of variance (ANOVA) and the Tukey test were used to determine whether significant differences existed for the laboratory-scale sewage treatment plants (LSSTPs) over the trial period. In addition to calculating the global significance values (p values), the data were analyzed pairwise using the Tukey test to deliver detailed significant differences between the different LSSTPs. Bonferroni corrections as a post test are not necessary.

Results

Technical Parameters of the Laboratory-Scale Sewage Treatment Plant (LSSTP)

During the test temperature, oxygen concentration, pH, dry matter, and dissolved organic carbon (DOC) elimination did not markedly differ between the laboratory-scale sewage treatment plants (LSSTPs). The temperature in all plants was very stable with an average temperature of 21°C and a standard deviation of 1.2°C. The average oxygen

Table 5 Isolated menaquinones

Main menaquinone components	Secondary menaquinone components	Bacterial strain	Reference
M 9(H_8), M 9(H_6)	M 9(H_2), M 8(H_8), M 8(H_6), M 8(H_4), M 9(H_2)	*Actinomadura madurae* (DSM 43067)	Tamaoka et al. (1983)
M 8(H_4)	M 7(H_4), M 6(H_4)	*Nocardia transvalensis* (DSM 43405)	Yamada et al. (1977)
M 9(H_2)	M 9, M 8(H_2), M 7(H_2)	*Arthrobacter globiformis* (DSM 20124)	Collins et al. (1979)
M 11, M 12	M 8, M 9, M 10, M 13	*Aureobacterium testaceum* (DSM 20166)	Collins et al. (1980)
M 7	M 8, M 6, M 5	*Staphylococcus epidermidis* (ATCC 12287)	Collins and Jones (1981)
M 8(H_2)	_	*Rhodococcus rhodochrous* (DSM 43241)	Collins et al. (1979)

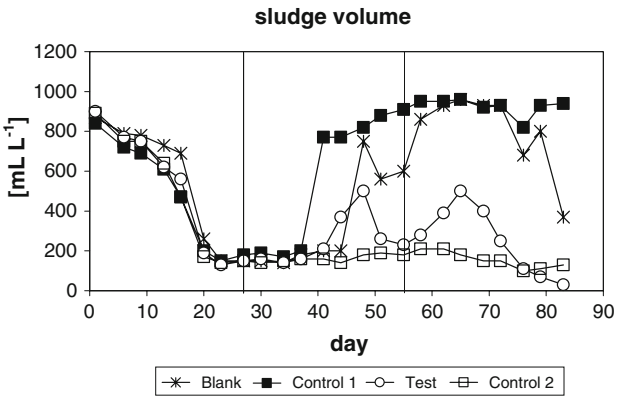

Fig. 1 Sludge volume during the trial

concentration in the aeration vessels was between 4.2 and 4.8 mg l^{-1} (standard deviation <1 mg l^{-1}), the pH was 7.5 (standard deviation 0.1) and the average dry substance lay between 2.0 and 2.6 g, with standard deviation below 1.0 g. Sludge age was 31 days. DOC elimination at the end of the test was at least 88% in all LSSTPs. According to the test guidelines, the tests were valid and the antibiotic mix did not affect DOC elimination. The sludge volume and the sludge volume indices of the plants that received the antibiotic mixture (control 2, test) significantly differed ($p \leq 0.05$) from the ones that received no antibiotic mixture (Fig. 1, Table 6). There was a significant difference in sludge volume of at least 300 ml l^{-1} at all measured points.

Effects on Sewage Bacteria

Colony-forming units (CFUs) monitoring showed that the number of bacteria able to grow on the medium optimized for sewage bacteria changed little during the whole trial. The antibiotics did not influence the presence of these bacteria. No significant differences between the CFU numbers of all laboratory-scale sewage treatment plants (LSSTPs) were detected on the growth medium without antibiotics compared with the medium with antibiotics ($p \geq 0.05$, Table 6). The introduction of *Acinetobacter baumannii* resulted in slightly elevated CFUs. After a few days, this effect was gone (Fig. 2). The monitoring of CFUs in each of the LSSTPs with the agar containing the mix of antibiotics to select for multiresistant bacteria showed that the total number of multiresistant CFUs is on average approximately two to three orders of magnitude lower than that of the non-multiresistant ones. The proportion of *A. baumannii* was high shortly after its addition, but was low again after only 1 week. The difference between the LSSTPs disappeared a few days after this event (Fig. 3). Other multiresistant CFUs were already present in the sewage sludge used in the LSSTPs, but were two to three orders of magnitude lower than those detected after the addition of multiresistant *A. baumannii* (Fig. 3), which increased slightly during the trial.

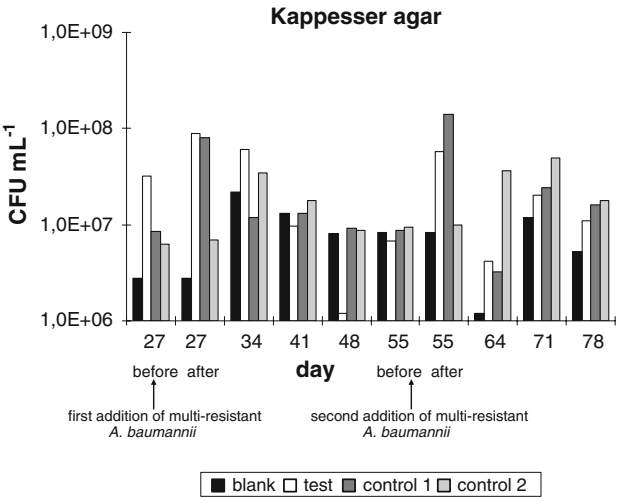

Fig. 2 Monitoring of all colony-forming units (CFUs) using Kappesser agar in the different laboratory-scale sewage treatment plants before and after the addition of multiresistant *Acinetobacter baumannii*. Antibiotics were delivered continuously at low concentration on days 27–55 and at high concentration on days 55–84 in the test plant and control 2

Table 6 Statistical comparison of all plants (Table 1) concerning colony-forming units (CFUs), dissolved organic carbon (DOC), and sludge volume indices (SVI) using ANOVA and Tukey test (SPSS 14.0)

	DOC	CFUs on growth broth without antibiotics	CFUs on growth broth with antibiotics	SVI
Blank and control 1	−	−	−	−
Blank and control 2	−	−	−	+
Blank and test	−	−	−	+
Control 1 and control 2	−	−	−	+
Control 1 and test	−	−	−	+
Control 2 and test	−	−	−	−

+ significant difference ($p \leq 0.05$)

− nonsignificant difference ($p \geq 0.05$)

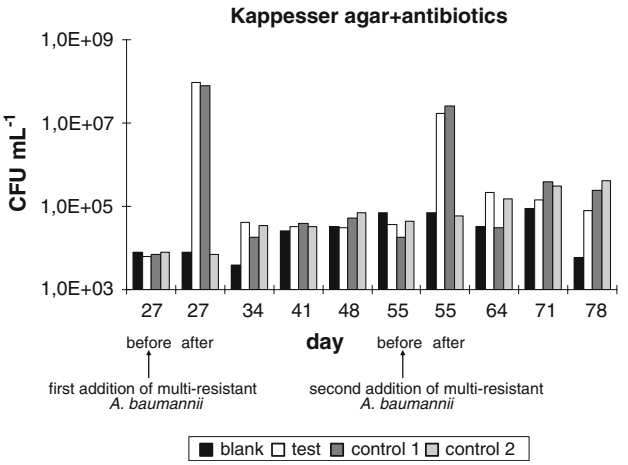

Fig. 3 Monitoring of multiresistant colony-forming units (CFUs) using Kappesser agar + antibiotics in the different laboratory-scale sewage treatment plants before and after the addition of multiresistant *Acinetobacter baumannii.* Antibiotics were delivered continuously in low concentration on days 27–55 and in high concentration on days 55–84 in the test plant and control 2

Chemotaxonomic profiling was performed using several compounds typical for different classes of bacteria. The main components of the polyamines were putrescine and sym-homospermidine in all the LSSTPs. At the beginning of the trial (acclimatization phase, first 3 weeks), the concentration of these compounds decreased and then increased again. The concentration of diaminopropane increased as expected shortly after the addition of *A. baumannii*. The concentrations of putrescine were significant different between control 1 and control 2 ($p \leq 0.05$, Tables 7 and 8). The difference in percentage of spermidine in the sludge of the plants that received the multiresistant bacterium and/or the antibiotic mixture (control 1, control 2, test) was statistically significant ($p \leq 0.001$, Tables 7 and 8) compared with the blank.

The main component of the ubiquinones was ubiquinone 8 (Q 8). Within the acclimatization phase (first 3 weeks) and the phase with low concentrations of antibiotics, no differences were found in ubiquinone patterns among the LSSTPs. The addition of the *A. baumannii* suspension led to a marginally elevated concentration of Q 9 each time. This effect disappeared each time after 1 week. Q 10 concentration increased after the elevation of antibiotic concentration in the test LSSTP which had received *A. baumannii.*

Within the adaptation phase (first 3 weeks), the concentration of the menaquinones was the same in all LSSTPs. Within the phase where the antibiotic mix was applied, the shares of menaquinone 7 (M 7) and menaquinone 8 (M 8) changed. This effect was stronger after the antibiotic concentration was increased. At several points during the trial, differences between the LSSTPs

Table 7 Results of all investigated chemotaxonomic parameters in the laboratory-scale sludge treatment plants (Table 1)

	CAD	DAP	HPUT	HSPD	PUT	SPD	M6	M7	M8	$M8H_2$	$M9(H_8)$	M9 & $M8(H_4)$	M10 & $M9(H_4)$	Q6	Q7	Q8	Q9	Q10
Blank	0.4 ± 0.2	4.6 ± 3.3	0.7 ± 0.4	5.9 ± 1.4	8.5 ± 3.2	2.8 ± 1.6	0.7 ± 0.2	5.2 ± 1.8	2.6 ± 1.2	0.4 ± 0.3	0.7 ± 1.3	0.6 ± 0.6	0.2 ± 0.2	0.02 ± 0.005	0.01 ± 0.006	15.7 ± 4.7	1.9 ± 0.7	4.7 ± 1.3
Control 1	0.4 ± 0.2	4.9 ± 4.3	0.6 ± 0.4	5.4 ± 1.7	8.7 ± 4.1	0.4 ± 0.2	0.8 ± 0.4	4.9 ± 1.9	2.5 ± 1.0	0.4 ± 0.3	0.7 ± 1.2	0.7 ± 1.3	0.7 ± 1.4	0.02 ± 0.008	0.01 ± 0.005	16.7 ± 6.2	2.2 ± 0.8	5.4 ± 1.6
Control 2	0.3 ± 0.1	3.2 ± 1.4	0.5 ± 0.1	5.4 ± 1.3	6.8 ± 1.5	0.3 ± 0.1	0.6 ± 0.2	3.8 ± 1.7	4.0 ± 2.1	0.4 ± 0.2	0.7 ± 1.4	0.2 ± 0.2	0.2 ± 0.2	0.02 ± 0.005	0.01 ± 0.005	15.3 ± 4.1	1.9 ± 0.5	5.5 ± 1.6
Test	0.4 ± 0.3	4.0 ± 3.0	0.6 ± 0.2	5.4 ± 1.9	7.9 ± 3.0	0.4 ± 0.3	0.8 ± 0.3	3.3 ± 1.5	3.5 ± 1.9	0.5 ± 0.3	0.7 ± 1.3	0.6 ± 0.5	0.6 ± 0.5	0.02 ± 0.006	0.01 ± 0.006	19.2 ± 7.8	2.4 ± 0.9	6.4 ± 2.8

Means and standard deviations (24 measurements during the trial) of μmol g^{-1} solid substance of activated sludge are given. *CAD* cadaverine, *DAP* diaminopropane, *HPUT* hydroxyputrescine, *HSPD* sym-homospermidine, *PUT* putrescine, *SPD* spermidine, *M* menaquinone, *Q* quinone

Table 8 Statistical comparison of all plants (Table 1) concerning all investigated chemotaxonomic parameters using ANOVA and Tukey test (SPSS 15.0)

	CAD	DAP	HPUT	HSPD	PUT	SPD	M10 & M9(H_4)	M6	M7	M8	M8(H_2)	M9(H_8)	M9 & M8(H_4)	Q6	Q7	Q8	Q9	Q10
Blank and control 1	−	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−
Blank and control 2	−	−	−	−	−	++	−	−	+	++	−	−	−	−	−	−	−	−
Blank and test	−	−	−	−	−	++	−	−	++	−	−	−	−	−	−	−	−	++
Control 1 and 2	−	−	−	−	+	−	−	−	+	++	−	−	−	−	−	−	−	−
Control 1 and test	−	−	−	−	−	−	−	−	++	+	−	−	−	−	−	−	−	−
Control 2 and test	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−

CAD cadaverine, *DAP* diaminopropane, *HPUT* hydroxyputrescine, *HSPD* sym-homospermidine, *PUT* putrescine, *SPD* spermidine, *M* menaquinone, *Q* quinone

\+ significant difference ($p \leq 0.05$)

++ highly significant difference ($p \leq 0.001$)

− nonsignificant difference ($p \geq 0.05$)

were observed. However, with the exception of M 7, statistical analysis of all chemotaxonomic biomarkers over the whole trial (ANOVA, SPSS 15.0, Table 8) showed no significant differences between the four LSSTPs which could be attributed to the addition of the antibiotic mixture. For M 7, significant differences between the blank and control 2, between the controls 1 and 2, and between the blank and the test LSSTP, as well as between control 1 and the test LSSTP were found. M8 only showed significantly different percentages between the blank and control 2, control 1, and control 2, and between control 1 and the test LSSTP. For Q 8 and Q 9 significant differences were determined between control 2 and the test LSSTP but not between the blank and the other LSSTPs. For Q 10 significant differences were detected only between the blank and the test LSSTP.

Discussion

We investigated the significance of the presence of a realistic mixture of antibiotics for the elimination of DOC and for possible effects on the population dynamics of resistant and nonresistant bacteria present in laboratory-scale sewage treatment plants (LSSTPs). Temperature, oxygen concentration, and DOC elimination of the four LSSTPs did not differ significantly during the entire test period. The antibiotic mix did not affect biodegradation performance (DOC elimination), which can be seen as a first hint that the impact on bacterial population dynamics by antibiotics was low. No significant differences were found in the number of colony-forming units (CFUs). On the Kappesser agar with antibiotics some bacteria had grown before antibiotics were supplied. Therefore, bacteria resistant to antibiotics were already present in the sludge. Only a few days after the batchwise addition of the multiresistant *Acinetobacter baumannii,* the level of CFUs was the same as before its addition. Though all sludge bacteria are in competition in LSSTPs, the addition of multiresistant *A. baumannii* had no competitive advantage from the presence of antibiotics in concentrations that were 10- to 100-fold higher than concentrations typically present in municipal sewage treatment plants (STPs). This should be noted, since multiresistant bacteria already present in the LSSTPs were at levels only two to three orders of magnitude lower than those of *A. baumannii* after addition. Neither *A. baumannii* nor the multiresistant bacteria present in the sewage sludge in general showed a selection advantage in the presence of the supplied antibiotics. This can be due to several reasons. It is possible that the antibiotic concentrations which were used were too low to cause a selective advantage for the resistant bacteria. Or, the antibiotics may not have penetrated into the activated sludge flocks, which are a complex of solitary cells embedded in extracellular polymeric substances. It is known that bacteria in biofilms are up to 500 times more resistant to antimicrobial agents than those growing in a planktonic state (Costerton et al. 1999). Another explanation could be that the antibiotics were rapidly eliminated. Findings on biodegradability testing of antibiotics have shown that these compounds are quite resistant to biodegradation by bacteria (Al-Ahmad et al. 1999; Alexy et al. 2004, Halling-Sørensen et al. 2000; Junker et al. 2006). For penicillins and cephalosporines and trimethoprime it has only recently been demonstrated that little elimination in LSSTPs takes place. These findings are in line with the low biodegradability of antibiotics seen in other test systems, as well as their presence in the effluent from LSSTPs (Junker et al. 2006), STPs and in surface water (Benito-Pena et al. 2006; Castiglioni et al. 2006; Cha et al. 2005). Furthermore, in LSSTP trials with similar setups using single antibiotics and without resistant *Acinetobacter*, it was

found that antibiotics which were present in the mixture at the same concentration range as those used in the current study were still present in the effluent (elimination rate 20–80%; Kümmerer et al. unpublished results).

In summary, one can assume that the presence of antibiotics in realistic concentrations, which were 10- to 100-fold smaller than the antibiotic concentrations which were tested here, do not result in an advantage for multiresistant *Acinetobacter* in sewage sludge.

The biomarkers used in this study allow for the monitoring of the effects on various important groups of sludge bacteria. The content of polyamines in Gram-positive bacteria is low. Therefore, in mixed cultures such as sewage sludge polyamines are useful for the monitoring of Gram-negative bacteria. Major components which were found in all four LSSTPs were putrescine and sym-homospermidine. Minor components were spermidine, hydroxyputrescine, and cadaverine. The analysis of polyamines in the activated sludge from the municipal STP where we sampled the sludge for the inoculation of the LSSTPs showed similar distribution patterns for the polyamines (data not shown). This indicates the acclimatization of the main bacterial group of municipal STP to the laboratory-scale environment. Ubiquinones are useful markers for Gram-negative bacteria which divide into several subclasses of proteobacteria. Q 8 is mainly present in proteobacteria of the β-subclass. Q 10 is a marker of the α-subclass, and Q 9 of the γ-subclass (Hiraishi et al. 1998). We found Q8 and Q10 to be major components. Q9 is an important component of the *Acinetobacter* species. Monitoring of the ubiquinones in the LSSTPs showed no significant difference which could be caused by adding the antibiotic mixture. The change was insignificant between the control and the test plant during the course of the test. Only the addition of *A. baumannii* resulted in an increase of ubiquinone Q 9.

Menaquinones are useful as biomarkers for Gram-positive bacteria. The main components in our trial were M 7 and M 8, which is in accordance with findings of Wiethan et al. (2001) for sewage sludge. They are markers for Gram-positive bacteria with low Guanin/Cytosin content. A number of different menaquinones were detected (Table 7). The major components were M 6, M 8(H_2), M 9(H_8), and M 9(H_2). The menaquinones M 9, M 8(H_4), M 10, and M 9(H_4) were minor components. In all LSSTPs the menaquinones M 9, M 8(H_4), M 10, M 9(H_4), M 9(H_2), and M 9(H_8) decreased continuously. The antibiotics did not cause the elimination of bacterial groups which significantly contribute to these menaquinones. This change was part of the acclimatization of the microbial biocenosis due to the constraints of the LSSTPs. The adding of the antibiotic mixture had an impact on the bacterial groups containing M 7. The concentration of this menaquinone changed significantly (Table 8). This is in accordance with the less complicated structure of cell envelope of Gram-positive bacteria making them more sensitive to antibiotics in general.

Sludge volume decreased within the acclimatization phase. During the phase when low antibiotic concentration was applied, it increased significantly in the two plants that received no antibiotics. However, this increase happened only 10 days after the continuous feeding with antibiotic mix had been started. According to our findings, Gram-positive bacteria probably caused this bulk sludge, since Gram-positive bacteria containing M 7 were affected by the antibiotic mixture. Nevertheless, in our experience such production of bulking sludge may also occur without a visible external trigger. This is true in both LSSTPs that receive antibiotics (or other chemicals) and in those that do not. Therefore, this difference is probably not of any significance. The used pilot sewage treatments plants should simulate the conditions present in municipal sewage treatment plants, where there are variations in a number of parameters. Such variations could not be excluded in a repetition of the test. Additionally, a repetition would not guarantee that the inoculum composition would be the same.

Biomarker monitoring and CFUs counting showed that the addition of a suspension of multiresistant *A. baumannii* resulted in an increase of multiresistant bacteria. This difference disappeared after 1–2 weeks. With the exception of the M 7 changes, neither the antibiotics, nor their possible aerobic degradation products, had any effect against the sludge. This corresponds to results obtained by Ohlsen et al. (1998), who found that resistance is not promoted even in the presence of the high antibiotic concentrations found in hospital effluent. *A. baumannii* is known to be present in the sewage of different STPs (Wiedmann-Al-Ahmad et al. 1994). Therefore, this bacterium should be able to survive and grow there. The multiresistant bacterium was not able to outcompete other bacteria even under strongly favorable conditions, i.e., in the presence of a mix of the most frequently used antibiotics at high concentration. Furthermore, it is generally assumed that bacteria resistant to antibiotics may have an advantage in competition with other bacteria. The results presented here show that, at least for the multiresistant *A. baumannii*, this assumption is not compelling. Nevertheless, studies on other resistant bacteria are required. However, the spread of antibiotic resistance genes is a consequence of intergeneric gene transfer coupled to the selective pressures posed by the overuse of antibiotics in human health care and animal feedstuffs (Davison 1999). Horizontal gene transfer can cause a spread of antibiotic resistance in different environments, especially in habitats with high bacterial density and diversity such as activated sludge. This could

prove to be clinically relevant with regard to pathogens (Martinez 2008).

Conclusion

Even when added at a high concentration, multiresistant *Acinetobacter baumannii* was not able to dominate other bacterial groups in the presence of several typical antibiotics in the laboratory-scale sewage treatment plants (LSSTPs) over a long period. Our results show that the presence of antibiotics even in microbiologically effective concentrations did not markedly influence the bacterial populations present in sewage treatment plants (STPs). According to the results presented here, one could assume that the input of already resistant bacteria is the main source for the resistant bacteria found in sewage treatment plants. This should be investigated for other groups of bacteria. If the main source of resistant bacteria stems from the medical use of antibiotics, then risk management should address this.

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