Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2018.22391

112 (2018) 250–257
April

Mapping of antibiotic resistant enterococci in wastewater treatment plants in Greece

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Received 30 January 2018; Accepted 20 April 2018

ABSTRACT

The widespread as well as the injudicious use of antimicrobial agents has generated an intense ecological imbalance through the emergence and spread of antibiotic resistant microorganisms, pathogenic or non-pathogenic. Agricultural use of sewage sludge bears numerous beneficial aspects, but it may also pose a potential threat for the environment and public health. In this study, potential environmental risks from sewage sludge application on soil are investigated, with emphasis on the spread of antibiotic resistant enterococci. Sixty-four samples of dewatered sludge from 56 wastewater treatment plants in Greece were analysed. The enterococcal density ranged from 1.8×10^3 to 1.14×10^7 cfu/g of dewatered sludge. The size of the enterococci population was not influenced by the size of the treatment plant. From the samples examined, 617 different *Enterococcus* spp. strains were isolated. Their antibiotic susceptibility was evaluated against 12 different antibiotics, representing a broad range of antimicrobial agents, using the disc diffusion method. The geographical distribution of antibiotic resistance demonstrated a widespread resistance to several antibiotics, including a substantial (14%) presence of multiresistance. Results demonstrate a high prevalence of antimicrobial agents' resistance in sewage sludge microorganisms. This may pose a threat to public health, if sludge is applied to the soil without some type of prior sanitisation.

Keywords: Antibiotic resistance; *Enterococcus* sp.; Sewage sludge

1. Introduction

During the past 50 years, the use and abuse of antimicrobial agents has led to an intense ecological imbalance through the emergence and spread of a large number of antibiotic resistant microorganisms, pathogenic or non-pathogenic [1]. This has been connected with the broadness of antibiotic applications on prevention and treatment of human disease, and consequently their widespread use in human medical practice, in animal husbandry and aquaculture [2–5]. Enterococci are commensals living in the intestinal tract of humans and animals, very often carrying resistance to a variety of drugs. The presence of antibiotic traces in human or animal gut may exert a selective pressure to these bacteria [6,7]. It has been proposed that the antibiotic resistance of these non-pathogenic bacteria isolated from different environments can give us an indication of the burden of antimicrobial resistance that may be present in a population [7]. For this reason, enterococci are very often used to measure the presence of antibiotic resistance [8–11]. Several antimicrobial resistance elements identified in enterococci are transferable over genus and species limits, as they are located on

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Presented at the 5th International Conference on Sustainable Solid Waste Management (ATHENS 2017), 21–24 June 2017, Athens, Greece.
1944-3994/1944-3986

conjugative or mobilisable plasmids and transposons, including genes encoding resistance to vancomycin, tetracycline and erythromycin [6,12].

Although there is no solid evidence that antibiotic resistance develops in wastewater treatment plants (WWTPs) [13], many studies state that sludge from WWTP constitutes a favourable medium for spreading resistant bacteria in the environment, as it consists of nutrients and bacteria of distinct faecal origin, which may act as a reservoir of antibiotic resistant genes with ideal conditions for both their survival and gene transfer [5,14–17]. According to literature, antibiotic resistant bacteria are found quite more frequently in wastewater biofilms than biofilms derived from surface water [18]. Antibiotic resistant enterococci may be transferred through the food chain to gut microbiota and subsequently to WWTP in a vicious cycle [4,7,14,16,19–22]. Though enterococci are not considered primary pathogens, they have been increasingly implicated in hospital infections worldwide [24,25], while gene transfer can easily occur between pathogenic and non-pathogenic bacteria [1,3,4,7,15].

Sustainable management of sewage sludge still constitutes a serious and multifaceted waste management issue, especially for medium-sized and smaller WWTPs, in which case thermal drying and incineration are normally unfeasible economically. Current EU policy and legislation, as expressed in the Sewage Sludge Directive 86/278/EEC and the Urban Waste Water Directive 91/271/EEC, amended by the 98/15/EC Directive, favour the beneficial agricultural use of sludge, provided that public health and environmental risks are minimised [25]. Moreover, the Landfill Directive 99/31/EC places restrictions on the amount of biodegradable waste allowed to landfills and demanding standards for landfill operation, both of which will eventually phase out landfilling of sewage sludge, currently accounting for 35%–45% of the sludge quantities produced in Europe [25,26].

Agricultural application of treated sludge from WWTPs, utilized as a soil conditioner, constitutes today its principal outlet in the EU (approximately 39%) and worldwide [25–27]. Landfilling is the second major route across EU, representing 37% of total sludge production. Incineration accounts for about 9% of sludge produced in the member states [26]. In Greece, however, only 10% of the sludge produced is utilised in agriculture, with landfilling still being the most commonly utilized option [28], while 'exported' incineration is practiced mainly for the sludge produced in the Psyttalia WWTP [29], that is, the sewage treatment plant which serves the greater metropolitan Athens region. Agricultural use of sludge bears numerous beneficial aspects and is generally compatible with the principles of sustainable development, namely waste recycling. It contributes to the enrichment of soil organic matter, the increase of agricultural production, the preservation of soil biodiversity, as well as to the reduction of chemical fertilizers use [25–26,30,31]. Nevertheless, the sludge is also likely to contain harmful and toxic compounds or pathogenic microorganisms [26,31], which constitute a potential threat for public health and the environment if they enter in the food chain [13,23,30,32,33]. Assessment of the hazard to humans and a rigorous set of regulatory and monitoring measures are required to assure safe use of sludge in agriculture, in order to gain the food supply chain's and consumer's acceptance [33].

The aim of this study was to assess the number of antibiotic resistant *Enterococcus* spp. present in sewage sludge from different WWTP in Greece, as this may pose serious risks to public health and the soil ecosystem.

2. Methodology

In this study, 64 samples of dewatered sludge have been collected, originating from 56 WWTPs all over Greece (see Fig. 1 and Table 1 for details). Samples were collected in sterile disposable vessels and transported to the laboratory (Harokopio University, Athens) for immediate processing (within 24 h since sampling time). Electrical conductivity of the samples ranged from 379 to 1,915 μS/cm (average: 666 μS/cm; standard deviation: ±356), while pH ranged from 6.2 to 7.8 (average: 7.0; standard deviation: 0.4). A composite sample of about 1 kg, consisting of four sub-samples of sludge (approximately 250 g each) was collected. The population of enterococci was measured, and the density per gram of dewatered sludge was estimated in the samples.

Enterococcus spp. strains were isolated in Slanetz–Bartley medium [24,35] and were characterised using morphological (gram-positive cocci) and biochemical tests (catalase – ability to grow at 10°C and at 45°C, in nutrient agar supplemented with 6.5% NaCl and in Bile Aesculin Agar) [35].

Antibiotic susceptibility of enterococci strains was tested in vitro by the agar disc diffusion technique in Mueller–Hinton agar (Oxoid), as recommended by the National Committee for Clinical Laboratory Standards [36,37]. Twelve different antimicrobial agents were applied in dried filter disc form, with the following potencies (BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs, Becton, Dickinson and Company [Sparks, MD 21152 U.S.A.]): ampicillin (AMP, 10 μg), bacitracin (BAC, 10 μg), ciprofloxacin (CIP, 5 μg), erythromycin (ERY, 15 μg), gentamycin (GEN, 120 μg), nitrofurantoin (NIT, 300 μg), norfloxacin (NOR, 10 μg), ofloxacin (OFX, 5 μg), rifampicin (RIF, 5 μg), streptomycin (STR, 300 μg), tetracycline (TET, 30 μg) and vancomycin (VAN, 30 μg). Isolates were characterised as either being susceptible, or intermediate or resistant, according to NCCLS zone diameter interpretive standards [36,37].

Comparison of populations of *Enterococcus* spp. in the sludge from different size WWTPs in Greece was performed by one-way analysis of variance (ANOVA) for parametric data, based on $\log_{10}$ transformation of viable bacterial counts. Normality of the distribution of enterococci counts was tested using the Kolmogorov–Smirnov test. Rates of antibiotic resistance of enterococci isolates depending on population size (number of inhabitants) of tested cities or presence of hospital sewage were compared by the chi-squared test (χ^2). The statistical analysis of the results was performed using the software programs Microsoft® Excel 2000 and SPSS® for Windows Release 11.5. A p-value less than 0.05 was considered to be the limit which denotes statistical significance.

3. Results and discussion

3.1. Quantification of Enterococcus spp. in sewage sludge

Sludge samples originating from 56 WWTPs, representing all the geographical departments of Greece, were examined for the presence of *Enterococcus* spp. All the samples were found positive. The enterococcal density ranged from 1.8×10^3 to 1.14×10^7 cfu/g of dewatered sludge. In order to examine the impact of the city's population size, on the enterococci population, cities were grouped based on their population as follows: (a) less than 10,000 people, (b) between 10,000 to 50,000 people and (c) more than 50,000 people. The results are presented in Table 2. There are no differences between the enterococci counts of the three groups of WWTPs ($p = 0.199$), suggesting that the size of the enterococci population was not influenced by the size of the treatment plant. In another survey for different bacterial groups in sludge from WWTPs in Germany, the mean value of enterococci was slightly lower (5.25 $\log_{10}$ cfu/g) compared with the values measured in this study [9]. Moreover, the number of enterococci measured in sludge from 14 towns in Portugal ranged from 7.0×10 to 3.0×10^7 cfu/g [8].

Novo and Manaia [10] report that the values of enterococci differ significantly among the three plants that they examined, presenting higher densities of enterococci in the treated wastewater (and not sludge) from the WWTP of the larger city.

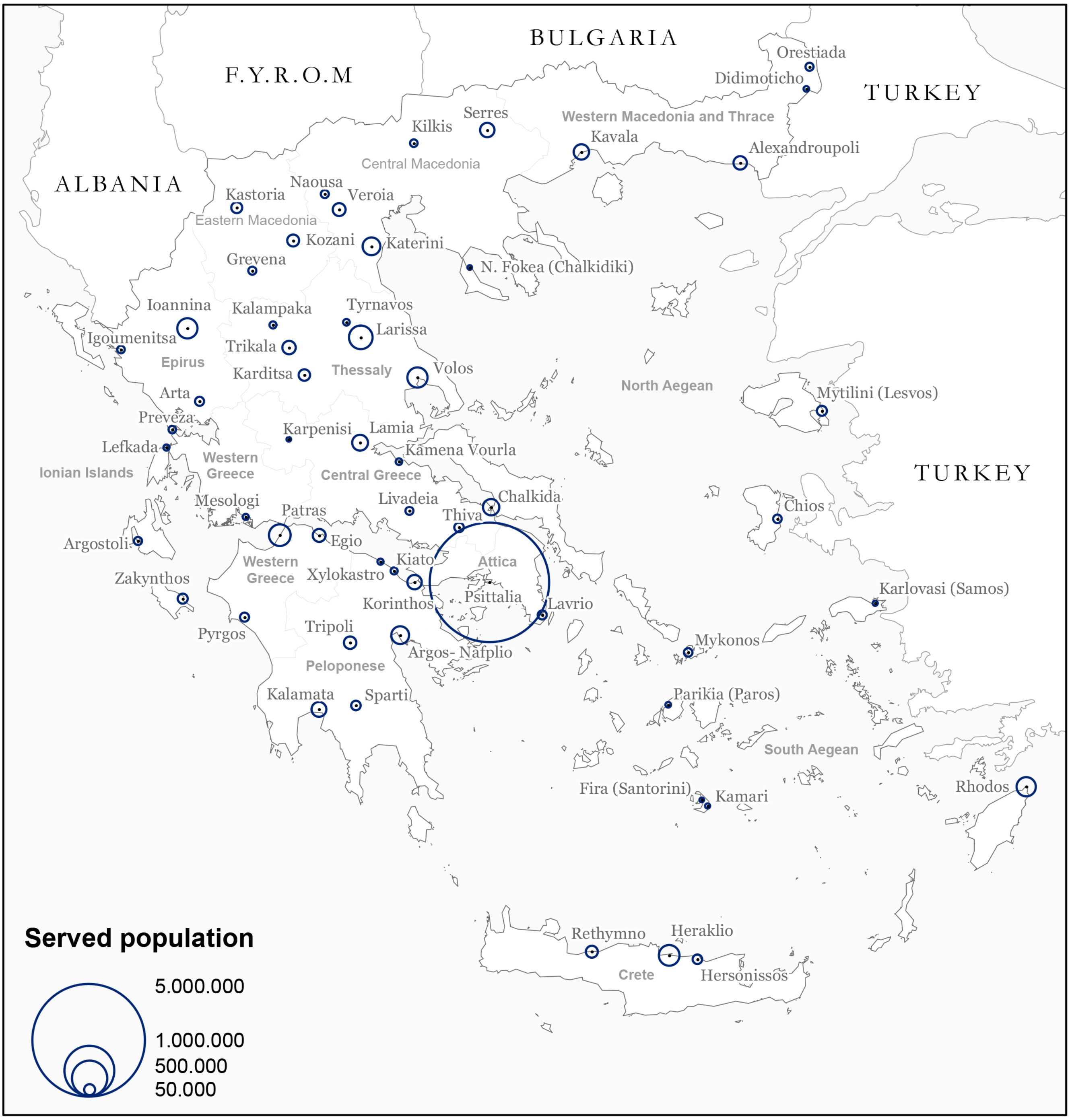

Fig. 1. Geographical distribution of sampling locations (wastewater treatment plants).

Table 1
Information on the wastewater treatment plants where sampling occurred

Water authority regions of Greece	Covered WWTPs per region (%)	WWTP	WWTP capacity – population equivalent (inh/d)	Daily mass load (average kg BOD_5/d)
Western Peloponnese	27.3	Kalamata	92,000	3,500
		Patras[a]	195,000	11,359
		Pyrgos	35,000	1,407
Northern Peloponnese	40,0	Argostoli (Kefalonia)	30,000	905
		Egio	70,000	1,450
		Kiato[a]	23,000	440
		Korinthos – Loutraki[a]	90,000	2,642
		Xylokastro[a]	17,500	522
		Zakynthos	43,333	1,650
Eastern Peloponnese	21.4	Argos–Nafplio[a]	133,333	6,009
		Sparti	40,000	1,159
		Tripoli	68,133	2,210
Western Central Greece	23.1	Karpenisi	10,000	515
		Lefkada	17,300	500
		Mesologi	17,500	–
Epirus	36.4	Arta	36,670	1,740
		Igoumenitsa	27,000	2,000
		Ioannina[a]	165,000	5,616
		Preveza	25,000	1,100
Attica	22.2	Lavrio	35,000	1,100
		Psittalia	5,630,000	195,600
Eastern Central Greece	16.7	Chalkida	111,600	3,462
		Thiva	40,000	602
		Kamena Vourla	20,000	326
		Lamia	104,200	3,790
		Livadeia	30,000	1,800
Thessaly	42.9	Kalampaka	21,666	610
		Karditsa[a]	56,050	3,000
		Larissa	227,500	8,265
		Trikala	75,000	3,120
		Tyrnavos	19,500	400
		Volos	170,000	8,455
Western Macedonia	41.7	Grevena	30,000	1,187
		Kastoria	50,000	1,825
		Kozani	60,000	1,590
		Naoussa	29,000	525
		Veria	69,700	2,791
Central Macedonia	7.7	Katerini	130,000	10,000
		Kilkis	26,000	1,550
		Nea Fokea (Chalkidiki)	10,000	–
Eastern Macedonia	12.5	Kavala	96,000	2,984
		Serres	88,000	4,156
Thrace	50.0	Alexandroupoli[a]	74,500	4,310
		Didimotiho	15,000	823
		Orestiada	30,000	915
Crete	16.7	Heraklio	177,567	11,120
		Hersonissos	40,000	700
		Rethymno	60,000	3,950

(*Continued*)

Table 1 (*Continued*)

Water authority regions of Greece	Covered WWTPs per region (%)	WWTP	WWTP capacity – population equivalent (inh/d)	Daily mass load (average kg BOD_5/d)
Aegean Islands	26.7	Chios	32,500	1,885
		Fira (Santorini)	10,833	532
		Kamari (Santorini)	13,500	106
		Karlovasi (Samos)	12,217	516
		Mykonos	32,000	–
		Mytilini (Lesvos)	43,300	1,370
		Parikia (Paros)	14,300	200
		Rhodos	154,683	7,560

Source: Special Secretariat of Water [34].
[a]Two samples were collected and analysed.

Table 2
Population of *Enterococcus* spp. in the sludge from different size WWTP in Greece

Population size of cities with WWTP	Number of cities	Enterococci (mean $\log_{10}$ cfu/g)
<10,000	14	6.05 ± 0.52
10,000–50,000	25	6.12 ± 0.96
>50,000	17	5.63 ± 1.06

3.2. Antimicrobial susceptibility

From the 56 WWTPs examined, 617 gram-positive catalase-negative cocci were isolated from 64 sludge samples. These cocci were characterised as enterococci, after being tested for their ability to grow at 10°C and at 45°C, in high-salt concentration and in the presence of bile salts. These strains of *Enterococcus* spp. were tested for their susceptibility against 12 different antimicrobial agents. The geographical distribution of antibiotic resistance demonstrates that all the samples tested were positive (100%) for resistance to rifampicin, 56 of them (96.6%) were positive for resistance against erythromycin, 42 were positive (72.4%) for tetracycline resistance, 34 for ofloxacin resistance (58.6%), 31 for ciprofloxacin resistance (53.5%) and 26 for norfloxacin resistance (44.8%).

Among the 617 enterococci tested, 163 isolates (26.4%) were found to be non-resistant (sensitive or intermediate) to all the antimicrobials, a fact that shows the dissemination of antibiotic resistance markers in the WWTPs in Greece. In a similar survey for antibiotic resistant enterococci from wastewater and sludge from 14 towns in Portugal, only 21.9% of the isolated strains were found susceptible to all the antibiotics tested [8]. Although results are not entirely comparable, as this study covers a somewhat larger and different array of antibiotics, it seems that resistance might be slightly less widespread in Greece.

Of all the strains examined in this study, 192 strains (31.1%) were resistant to one antibiotic only, 127 (20.6%) showed resistance against two antibiotics and 48 (7.8%) were resistant against three antibiotics. Eighty-seven strains (14.1%) were defined as multiresistant (presenting resistance to more than three antibiotics). The most common simultaneous resistance against two antibiotics was for erythromycin and rifampicin (20.8%), in accordance to the findings of dendrogram analysis of resistance profiles of streptococci isolated from WWTPs in Portugal [8]. Among these multiresistant strains, 38 were resistant to four antibiotics, 14 were resistant to five antibiotics, 11 strains exhibited resistance against six antibiotics, 12 strains to seven antibiotics, 10 strains to eight antibiotics and finally two strains were found resistant against nine antibiotics.

Notwithstanding the variety of resistotypes in the multiresistant strains was very high, some trends about the frequency of the same core of antibiotic resistances can be identified:

- Fifty-two (representing 59.7% of the total) multiresistant strains had the same core of resistances against rifampicin, ciprofloxacin and one of the fluoroquinolones (ofloxacin or norfloxacin) (RA, CIP, OFX or NOR). Twenty-seven strains had this core of resistances and additionally resistance to erythromycin (RA, CIP, OFX or NOR, E). Nineteen strains out of 87 multiresistant presented resistance to ampicillin also (RA, CIP, OFX or NOR, E, AMP), fifteen strains had resistance to streptomycin (RA, CIP, OFX or NOR, E, S) and only eight strains to tetracycline (RA, CIP, OFX or NOR, E, TE). The first core of resistances (RA, CIP, OFX or NOR) was also associated with resistance to nitrofurantoin for 19 strains (RA, CIP, OFX or NOR, F/M) and 7 strains had additionally to this resistotype, resistance also against erythromycin.
- Thirty-three (or 37.9% of the total) multiresistant strains had the same core of resistances against rifampicin, ciprofloxacin and erythromycin

Rifampicin resistance was the highest observed, at 54.5%, a value slightly higher than the one (51.5%) observed in 983 strains from 14 WWTPs in Portugal [8]. Rifampicin is exclusively used in human medical practice, mainly to treat tuberculosis [38]. The striking high resistance to rifampicin may reflect the ecological dissemination of antimicrobial resistance through the innumerable pathways that allow to resistant bacteria and resistant genes to spread among animals and humans. Long-term exposure of bacteria to antibiotics may create such a stable resistance that the resistant strains can compete with the susceptible ones even in the absence of antibiotics [38]. Overall, resistance to antimicrobials acting as nucleic acid synthesis inhibitors was high: apart from rifampicin, a high prevalence of resistance to ciprofloxacin (19.8%), ofloxacin (14.3%) and norfloxacin (9.7%) was also detected. Resistance to ciprofloxacin was higher compared with the Portuguese study (13.9%). The same researchers suggest that the ciprofloxacin resistance prevalence in enterococci of food, animal and environmental origin is at similar levels to those observed in clinical isolates [8].

Moreover, a high prevalence of resistance to tetracycline (15.2%) and erythromycin (26.7%) was detected. Similar levels of high erythromycin resistance (24.8%) were detected in sludge samples from different towns in Portugal, an observation that might be coherent with the previous extensive use of macrolides (azithromycin and clarithromycin), in this country [8]. In Greece, there is also a widespread use of macrolides [39], which may explain the high prevalence of resistance. Frequent erythromycin resistance is probably related to the extended use of the macrolide tylosin for animal growth promotion [2,40]. In a recent survey for antibiotic resistant enterococci in the faecal microflora of non-hospitalised and even more, never medicated with antibiotics infants in Greece, the erythromycin resistance was 35.1% and was associated mainly with *E. faecium* isolates [41]. Erythromycin resistance in enterococci is mainly associated with the presence of *ermB* gene on the conjugative transposon Tn*917*, a transposable element that can easily move between different bacterial cells [42,43].

The frequency of tetracycline resistance found in this study was 15.2%, less than half of that found in wastewater and sludge isolates in Portugal (34.6%), possibly due to the more extensive use of tetracyclines in the animal husbandry practices in Portugal [8]. However, much higher prevalence of oxytetracycline resistance in enterococci from the faecal microbiota of healthy Greeks has been found in other studies (88.0%), possibly connected with the extensive use of these antibiotics in clinical practice [39]. According to the latest data on antibiotic consumption in European Union, in 2015, Greece was on the top of antibiotic consumption within the EU member states [44]. More specifically, the consumption in the community was 36.1 daily-defined dosage per 1,000 habitants per day [44]. The much lower resistance frequency in the sludge, found in this study, compared with the intestinal track of individuals is a result that deserves further research.

Resistance to ampicillin (5.2%) and streptomycin (6.2%) was relatively limited, in accordance to the results of the Portuguese study [38] and in contrast to the widespread use of these antibiotics in both countries. A possible explanation could be a particularly low capacity of resistant to these antibiotics enterococci for epidemic spread, and therefore their scarcity in sludge. However, this hypothesis demands further investigation. Ampicillin resistance was accompanied mostly with other resistances.

Only four strains (0.7%) were resistant to vancomycin, a very important antibiotic that is often used as the drug of last resort in treatment of infections caused by antibiotic resistant gram-positive bacteria [24]. However, this low-resistance findings should be interpreted with caution, as isolation and characterisation studies may mask the presence of other important sub-populations that may be more capable of growing within hosts than the selective growing media [8].

The influence of the city population to the prevalence of antibiotic resistance was also examined. The results are presented in Table 3. From all the antibiotics tested, only the rate of nitrofurantoin resistance is lower to small cities (<10,000 inhabitants), compared with more crowded ones (10,000–50,000 inhabitants p = 0.004 or >50,000 inhabitants p = 0.003). These results deserve further investigation, because furaltadone a chemical analogue of this antibiotic is used in poultry production [8]. Probably, it is worthy to examine which of these WWTPs accept wastewater from poultry farms and investigate if there are any differences in nitrofurantoin resistance.

The impact of the presence of hospital sewage in the WWTP on the prevalence of antibiotic resistance was also investigated. The results are presented in Table 4. From the 12 antibiotics tested, only one (bacitracin) seems to be influenced by the presence of hospital sewage.

The hypothesis that hospital wastewater entering the WWTP of the city without any previous treatment would enrich the sludge with antibiotic resistant strains does not seem to be verified from the results of this study. On the contrary, Martins da Costa et al. [8] suggest that the prevalence of different antibiotic resistances (vancomycin, erythromycin, ciprofloxacin, ampicillin, tetracycline and gentamicin) is influenced by the presence of hospital sewage. Novo and Manaia [10] found that the density of antibiotic resistant bacteria in the raw inflow of domestic sewage (and not in the sludge) was not significantly different from the observed in the plants receiving industrial or hospital effluents. The issue of grouping the WWTPs based on the presence of sewage from animal husbandry farms and its impact on the rates of antibiotic resistance should be further investigated.

Table 3
Rates of antibiotic resistance[a] depending on population size (number of inhabitants) of tested cities

	Population size						
	<10.000[b]		10.000–50.000[c]		>50.000[d]		p
AMP-10	5	(3.47)	17	(6.88)	10	(4.42)	0.276
B-10	5	(3.47)	15	(6.07)	12	(5.31)	0.532
CIP-5	22	(15.28)	57	(23.08)	43	(19.03)	0.164
E-15	44	(30.56)	68	(27.53)	53	(23.45)	0.302
GM-120	6	(4.17)	7	(2.83)	4	(1.77)	0.388
F/M-300	4	(2.78)	27	(10.93)	26	(11.50)	0.009
NOR-10	12	(8.33)	30	(12.15)	18	(7.96)	0.251
OFX-5	16	(11.11)	45	(18.22)	34	(15.04)	0.168
RA-5	86	(59.72)	141	(57.09)	115	(50.88)	0.198
S-300	12	(8.33)	16	(6.48)	9	(3.98)	0.210
Te-30	22	(15.28)	33	(13.36)	39	(17.26)	0.500
Va-30	1	(0.69)	2	(0.81)	2	(0.88)	0.980

[a]Values express numbers of resistant isolates, and numbers in parentheses express rates (%) of antibiotic resistance.
[b]14 cities and 144 isolates.
[c]26 cities and 247 isolates.
[d]8 cities and 226 isolates.
AMP-10, ampicillin 10 μg; B-10, bacitracin 10 IU; CIP-5, ciprofloxacin 5 μg; E-15, erythromycin 15 μg; GM-120, gentamycin 120 μg; F/M-300, nitrofurantoin 300 μg; NOR-10, norfloxacin 10 μg; OFX-5, ofloxacin 5 μg; RA-5, rifampicin 5 μg; S-300, streptomycin 300 μg; Te-30, tetracycline 30 μg; Va-30, vancomycin 30 μg.

Table 4
Rates of antibiotic resistance[a] depending on presence of hospital sewage in the tested cities

	Hospital sewage				
	No[b]		Yes[c]		*p*
AMP-10	9	(4.62)	23	(5.45)	0.644
B-10	4	(2.05)	28	(6.64)	0.017
CIP-5	35	(17.95)	87	(20.62)	0.439
E-15	59	(30.26)	106	(25.12)	0.180
GM-120	7	(3.59)	10	(2.37)	0.389
F/M-300	14	(7.18)	43	(10.19)	0.230
NOR-10	22	(11.28)	38	(9.00)	0.375
OFX-5	28	(14.36)	67	(15.88)	0.627
RA-5	117	(60.00)	225	(53.32)	0.121
S-300	14	(7.18)	23	(5.45)	0.400
Te-30	32	(16.41)	62	(14.69)	0.581
Va-30	1	(0.51)	4	(0.95)	0.575

[a]Values express numbers of resistant isolates, and numbers in parentheses express rates (%) of antibiotic resistance.
[b]18 cities and 195 isolates.
[c]38 cities and 422 isolates.
AMP-10, ampicilllin 10 μg; B-10, bacitracin 10 IU; CIP-5, ciprofloxacin 5 μg; E-15, erythromycin 15 μg; GM-120, gentamycin 120 μg; F/M-300, nitrofurantoin 300 μg; NOR-10, norfloxacin 10 μg; OFX-5, ofloxacin 5 μg; RA-5, rifampicin 5 μg; S-300, streptomycin 300 μg; Te-30, tetracycline 30 μg; Va-30, vancomycin 30 μg.

4. Conclusions

This study documents the presence of numerous multiresistant enterococci in sewage sludge from WWTPs in Greece. The results indicate that certain antibiotic resistance markers such as rifampicin and erythromycin are widely disseminated in all the WWPT examined. The highest rates were recorded for resistance to rifampicin, erythromycin, fluoroquinolones and tetracycline. These enterococcal strains need to be further examined in order to evaluate if they carry acquired or intrinsic antibiotic resistant markers. Moreover, the potential public health risks of antibiotic resistance from sludge spreading on soil should be studied and assessed versus other sources of antibiotic resistant enterococci, such as animal husbandry.

Acknowledgements

This research was partly supported by (a) the MSc Programme "Sustainable Development", Department of Home Economics and Ecology, Harokopio University and (b) the MSc Programme "Applied Geography and Spatial Planning", Department of Geography, Harokopio University. Authors would also like to thank Jenny Aggelopoulou (Harokopio University, Kallithea, Greece) and Tobias Rocktäschel (Bauhaus University, Weimar, Germany) for their valuable contribution to the conducted microbiological analyses.

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