# Antibiotics resistance in sewage sludge enterococci

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**Abstract**

In the last 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 antibiotics resistant microorganisms, pathogenic or not. 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 that may be found in sludge. 66 samples of dewatered sludge from 58 Wastewater Treatment Plants in Greece were analysed. The enterococcal density ranged from $1.8x10^3$ to 1.14 $x10^7$ cfu/g of dewatered sludge. It was found that the size of the enterococci population was not influenced by the size of the treatment plant (city population). 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 multi-resistance (resistance to more than three antibiotics.

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 sanitisation. Further research is required in order to access both: (a) the relevant risk from sludge application to soil compared to indigenous soil microbial population resistance, especially where manure is applied; and (b) the effect of different sludge treatment methods on antibiotics resistance.

## 1. Introduction

In the last 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 antibiotics resistant microorganisms, pathogenic or not [1]. This has been connected with the widespread use of antibiotics in human clinical practice, but also in animal husbandry [2,3] and aquaculture [4]. Enterococci are commensals living in the intestinal tract of humans and animals, carrying very often resistance to a variety of drugs. The presence of antibiotics traces in human or animal gut may exert a selective pressure to these bacteria [5,6]. 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 [5] and this is the reason that enterococci are very often used to measure the presence of antibiotic resistance [7-9]. Several antimicrobial resistance elements identified in enterococci are transferable over species and genus borders, as they are located on conjugative or mobilisable plasmids and transposons, including genes encoding resistance to vancomycin, tetracycline and erythromycin [6].

### 1.1. Background

The sludge from WTPs constitute a favourable environment 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 antibiotics resistant genes with ideal conditions for both their survival and gene transfer [10-12]. From there, resistant enterococci may find a way of returning to humans, either through water contamination [5,13] or through the food chain [4,10, 12-16]. Though enterococci are not considered primary pathogens, they have been increasingly implicated in hospital infections worldwide [17,18], while gene transfer can easily occur between pathogenic and non-pathogenic bacteria [1,2,4,5,11].

Sustainable management of sewage sludge still constitutes a serious and multifaceted waste management issue, especially for medium and smaller Wastewater Treatment Plants (WTP), whereas thermal drying and incineration is commonly economically unfeasible. 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 [18]. 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 [18,19].

Agricultural application of sludge, to be exploited as a soil conditioner, constitutes today its principal outlet in the EU (about 38%) and worldwide [18,19]. Landfilling is the second major route, representing 37 % of total sludge production. Incineration accounts for about 9% of sludge produced in the Member States [19]. In Greece, however, only 10% of the sludge produced is utilised in agriculture, with landfilling still being the most widespread option [20] and incineration practiced mainly for the sludge produced in the Psyttalia WTP of Athens. 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 the reduction of chemical fertilizers use [19-22]. Nevertheless, the sludge is also likely to contain harmful and toxic compounds or pathogenic microorganisms [19,21] which constitute a potential threat for public health and the environment if they enter the food chain [21,23,24]. Hazard to man and a rigorous set of regulatory and monitoring measures is required to assure safety and gain the food market and consumer's acceptance [24].

### 1.2. Research objectives

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

## 2. Methodology

In this study 66 samples of dewatered sludge have been collected from 58 WTP in Greece, during two dinstict periods: the first was from 2005 to 2006 and the second was from December 2007 to June 2008. More specifically, samples were collected from the WTP of Alexandroupoli (2 samples in two different time periods), Didimoticho, Orestiada, Kavala, Seres, Grevena, Klikis, Kastoria, Kozani, Katerini, Naousa, Veria, Chalkidiki (N. Fokea), Ioannina (2 samples in two different time periods), Arta, Karpenisi, Preveza, Igoumenitsa, Mesologi, Kalambaka, Larissa, Tyrnavos, Trikala, Karditsa (2 samples in two different time periods), Volos, Livadia, Thiva, Lamia, Kamena Vourla, Chalkida, Laurio, Athens, Korinthos-Loutraki (2 samples in two different time periods), Kiato (2 samples in two different time periods), Xylocastro (2 samples in two different time periods), Egio, Patras (2 samples in two different time periods), Argos, Nafplio (2 samples in two different time periods), Kalamata, Sparti, Pyrgos, Tripoli, Zakinthos, Argostoli (Kefalonia), Lefkada, Mykonos, Parikia (Paros), Fira (Santorini), Kamari (Santorini), Mytilini, Chios, Rodos, Karlovasi (Samos), Creta (Chersonissos, Iraklion, Rethymno). Samples were collected in sterile disposable vessels and transported to the laboratory (Harokopio University, Athens) for immediate processing (within 24 hours from collection). A composite sample of about 1 kg, consisting of four sub-samples of sludge (about 250 g each) was collected.

The population of enterococci was measured in the samples and the density per gr of dewatered sludge was estimated in the samples. *Enterococcus* spp. strains were isolated in Slanetz-Bartley medium [17,25] and were characterised using morphological (Gram$^+$ cocci) and biochemical tests (catalase - ability to grow at $10^0$C and at $45^0$C, in nutrient agar supplemented with 6.5% NaCl and in Bile Aesculin Agar) [25].

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 [26,27]. Twelve different antimicrobial agents were applied in dried filter disc form, with the following potencies (BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs, Becton Dickinson): ampicillin (AMP, 10 μg), bacitracin (BAC, 10μg), 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 susceptible, intermediate or resistant according to NCCLS zone diameter interpretive standards [26,27].

## 3. Results and Discussion

### 3.1 Quantification of *Enterococcus* spp. in sludge

Sludge originated from 58 WTP, from all the geographical departments of Greece, was examined for the presence of *Enterococcus* spp. and all the samples were found positive. The enterococcal density ranged from $1.8x10^3$ to $1.14 x10^7$ cfu/g of dewatered sludge. In order to examine the impact of the city' s population size, to the enterococci population, cities were grouped according to their population to: lower than 10,000 people, ranged from 10,000 to 50,000 people and higher than 50,000 people. The results are presented in Table 1. There are not differences between the enterococci counts of the three groups of WTP (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 of WTP in Germany the mean value of enterococci was slightly lower (5.25 log cfu/g) than the values measured in the present study [8]. The number of enterococci measured in sludge from 14 towns of Portugal, ranged from 7.0x10 to $3.0x10^7$ cfu/g [7].

Novo and Manaia [9] refer 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 WTP of the bigger city.

**Table 1. Population of Enterococcus spp. in the sludge from different size WTP in Greece**

| Population size of cities with WTP | Number of cities | Enterococci (mean log cfu/g) |
|---|---|---|
| <10,000 | 14 | 6.05 ± 0.52 |
| 10,000-50,000 | 26 | 6.12 ± 0.96 |
| >50,000 | 18 | 5.63 ± 1.06 |

### 3.2 Antimicrobial susceptibility

From the 58 WTP examined, 617 Gram positive, catalase negative cocci isolated from 66 sludge samples, were characterised as enterococci, after being tested for their ability to grow at $10^0$C and at $45^0$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 from the 58 WTP 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 not resistant (sensitive or intermediate) to all the antimicrobials, a fact that shows the dissemination of antibiotic resistance markers in the WTP of Greece. In a similar survey for antibiotics resistant enterococci from wastewater and sludge from 14 towns in Portugal, only the 21.9% of the isolated strains were found susceptible to all the antibiotics tested [7]. Although results are not entirely comparable, as the current 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 the current 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. 87 strains (14.1%) were defined as multi-resistant (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 WTP in Portugal [7]. Among these multi-resistant strains, 38 were resistant to 4 antibiotics, 14 were resistant to 5 antibiotics, 11 strains exhibited resistance against 6 antibiotics, 12 strains to 7 antibiotics, 10 strains to 8 antibiotics and finally 2 strains were found resistant against 9 antibiotics.

Notwithstanding the variety of resistotypes in the multi-resistant strains was very high, some observations about the frequency of the same core of antibiotic resistances can be made:

i) 52 (59.7%) multi-resistant strains had the same core of resistances against rifampicin, ciprofloxacin, and one of the fluoroquinolones (ofloxacin or norfloxcin) (RA, CIP, OFX or NOR). 27 strains had this core of resistances and additionally resistance to erythromycin (RA, CIP, OFX or NOR, E). 19 strains out of 87 multiresistant, presented resistance to ampicillin also (RA, CIP, OFX or NOR, E, AMP), 15 strains had resistance to streptomycin (RA, CIP, OFX or NOR, E, S) and only 8 strains to tetracycline (RA, CIP, OFX or NOR, E, TE). The first core of resistances (RA, CIP, OFX or NOR) was also associated with resitance to nitrofurantoin for 19 strains (RA, CIP, OFX or NOR, F/M) and 7 strains had additionally to this resistotype, resistance also against erythromycin.

ii) 33 (37.9%) multi-resistant 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 that observed in 983 strains from 14 WTP in Portugal (51.5%). Rifampicin is exclusively used in human medicine, mainly to treat tuberculosis [28]. 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 between animals and humans. Long-term exposure of bacteria to antibiotics may create resistance so stable that the resistant strains can compete with the susceptible ones even in absence of antibiotics [28]. 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 than the Portuguese study (13.9%). The same researchers suggest that the ciprofloxacin resistance prevalence in enterococci of food, animal and environment origin is at similar levels to those observed in clinical isolates [7].

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, clarithromycin), in this country [7]. In Greece there is also a widespread use of macrolides [29], 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 [3,30]. 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 [31]. 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 [32,33].

The frequency of tetracycline resistance in this study was less than half of that found in wastewater and sludge isolates in Portugal (34.6% vs 15.2% in Greece), possibly due to the more extensive use of tetracyclines in the animal production in this country [7]. 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 [29]. The much lower resistance frequency in the sludge, found in the current study, compared to the intestinal track of individuals is a finding deserving further research.

Resistance to ampicillin (5.2%) and streptomycin (6.2%) were relatively limited, in accordance to the results of the Portuguese study [28] 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 [17]. 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 [7]. The influence of the city population to the prevalence of antibiotic resistance was also examined. The results are presented in Table 2. From all the antibiotics tested, only the rate of nitrofurantoin resistance is lower to small cities (<10,000 people), compared to the bigger cities (10,000-50,000 $p=0.004$ or >50,000 people $p=0.003$). These results deserve further investigation, because furaltadone a chemical analogue of this antibiotic is used in poultry production [7]. Probably, it is worthy to examine which of these WTP 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 treatment plant on the prevalence of antibiotic resistance was also investigated and the results are presented in Table 3. From the 12 antibiotics tested, only one (bacitracin) seems to be influenced by the presence of hospital sewage.

**Table 2: Rates of antibiotic resistance[1] depending on population size (number of inhabitants) of tested cities**

| | Population size | | | | | | |
|---|---|---|---|---|---|---|---|
| | **< 10.000[a]** | | **10.000 - 50.000[b]** | | **> 50.000[c]** | | ***p-value*** |
| **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 |

[1] Values express numbers of resistant isolates and numbers in parentheses express rates (%) of antibiotic resistance
[a] 14 cities and 144 isolates; [b] 26 cities and 247 isolates; [c] 8 cities and 226 isolates
AMP-10, Ampicillin 10μg; B-10, Bacitracin 10IU ; 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 5μg; RA-5, Rifampicin 5 μg; S-300, Streptomycin 300 μg; Te-30, Tetracycline 30μg; Va-30, Vancomycin 30 μg

The hypothesis that the hospital wastewater, ending to the WTP of the town without any previous treatment, would enrich the sludge with antibiotic resistant strains doesn't seem to be verified from the results of this study. On the contrary, Martins da Costa et al. [7] 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 [9] 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 WTP according to the presence of sewage from animal husbandries and its impact to the rates of antibiotic resistances should be further investigated.

**Table 3: Rates of antibiotic resistance1 depending on presence of hospital sewage in the tested cities**

| | Hospital sewage | | | | |
|---|---|---|---|---|---|
| | no[a] | | yes[b] | | *p-value* |
| 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 |

[1] Values express numbers of resistant isolates and number in parentheses express rates (%) of antibiotic resistance
[a] 19 cities and 195 isolates; [b] 39 cities and 422 isolates
AMP-10, Ampicilllin 10μg; B-10, Bacitracin 10IU; CIP-5, Ciprofloxacin 5μg; E-15, Erythromycin 15μg; GM-120, Gentamycin 120μg; F/M-300 Nitrofurantoin 300μg; NOR-10, Norfloxacin 10μg; OFX-5, Ofloxacin 5μg; RA-5, Rifampicin 5μg; S-300, Streptomycin 300μg; Te-30, Tetracycline 30μg; Va-30, Vancomycin 30μg

## 4. Conclusions

This study documents the presence of numerous multiresistant enterococci in sewage sludge from WTP from Greece. Especially certain antibiotic resistance markers, like rifampicin and erythromycin are widely disseminated in all the WPTs examined. The highest rates were recorded for rifampicin, erythromycin, fluoroquinolones and tetracycline resistances. 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 streptococci, such as the animal husbandry.

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