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Distribution and persistence of fecal bacterial populations in liquid and dewatered sludge from a biological treatment plant

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The changes in composition and structure of fecal coliforms (FC) and enterococci (ENT) populations, as well as the elimination of spores of sulphite-reducing bacteria (SRB), were compared between municipal sewage and their derived sludge in a biological treatment plant in order to determine any selective reduction or adsorption to sludge during the treatment process. Additionally, the persistence of antibiotic-resistant enterococcal populations in two kinds of sludge was also considered to evaluate their potential elimination in the treatment process. Microbial indicators, vancomycin-resistant and erythromycin-resistant enterococci were enumerated. The structure and composition of FC and ENT populations were determined by biochemical fingerprinting and clustering analyses. Raw and treated sewage showed a concentration of FC 1 log unit higher than ENT and nearly 2 log units higher than spores of SRB. However, the three studied indicators showed similar concentrations in both types of sludge. Consequently, FC were eliminated in higher proportion than ENT and spores of SRB in sludge. FC and ENT populations showed high diversity and similarity population indexes for all kinds of samples. Antibiotic-resistant enterococci persisted in a similar proportion in respect to total enterococci not only in treated sewage but also in sludge. The persistence of antibiotic-resistant strains in sludge as well as in treated sewage should be considered if they are used for land disposal or for water reutilization, respectively.

Key words——antibiotic resistance; bacterial diversity; clostridia; enterococci; fecal coliforms; sewage; sludge

Introduction

The ecology of microbial populations in water and wastewater environments has been extensively studied (Barcina et al., 1997; Scott et al., 2002; Sinton et al., 1994). Commonly, the reduction of fecal indicator bacteria is attributed to biotic and non-biotic factors such as predation, nutrient scarcity, temperature, osmotic stress and visible light. But these factors could affect these bacterial populations to different degrees. For instance, the survival of *Clostridium perfringens* is higher than that of fecal coliforms or enterococci in river waters and in swine wastewater (Hill and Sobsey, 1998; Medema et al., 1997). Gram negative bacteria are selectively preyed upon by ciliates (González et al., 1990). It has also been reported that in sewage treatment plants, bacteria are removed by inactivation, grazing by ciliated protozoa and adsorption to sludge solids and/or encapsulation within sludge flocks followed by sedimentation (Bitton, 1994). Attending to these complex processes of depuration, it should be expected that the predominant species or strains of different microbial indicators and their proportions in treated sewage would change in respect to raw

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sewage. However, it has been reported that in some treatment plants, the proportions of fecal coliforms (FC), enterococci (ENT) (Hill and Sobsey, 1998) and even in some cases spores of sulphite-reducing bacteria (SRB) (Vilanova et al., 2004) remained identical in treated sewage. It has been demonstrated that FC and ENT populations have high similarity in composition and structure between raw and treated sewage. Moreover, the predominant species of both bacterial groups are consequently the same (Vilanova et al., 2004) for both kind of sewages. In contrast, a different proportion of these bacterial indicators has been reported when comparing sludge with respect to sewage (Chauret et al., 1999). However, the diversity of FC and ENT populations in sludge has not been analyzed.

The water cycle may act as the transmission chain for some resistance to antibiotics such as vancomycin or erythromycin (Kühn et al., 2000). Vancomycin-resistant enterococci (VRE) have been identified as an important cause of hospital-acquired infection (Goossens, 1998) and have been related to animal production (Aarestrup, 1995; Klare et al., 1995; Robredo et al., 1999; Stobbering et al., 1999; Teuber and Perreten, 2000). Enterococci isolates from animal and human origins have also been reported to carry erythromycin resistance genes (Jackson et al., 2004; Jensen et al., 1999). Consequently, resistance to these two antibiotics could be potentially transmitted through the bacterial population of wastewater and sludge, particularly in sewage treatment plants which have a high concentration of bacteria of distinct fecal origin. The reutilization of treated sewage or sludge could carry a possible health risk if these antibiotic-resistant bacterial populations persist. The persistence of these VRE and ERE strains in treated sewage has already been demonstrated in previous studies (Vilanova et al., 2004). However, their accumulation or persistence in sludge of the treatment plants is poorly described.

In this study, the structure, diversity and composition of fecal coliforms and enterococci in sludge derived from a sewage treatment plant have been analyzed. These populations have been compared and related with those in raw and treated sewage. The accumulation and persistence of VRE and ERE in two kinds of sludge (liquid and dewatered) have been determined and compared with the rest of the bacterial indicators in the treatment plant.

Materials and Methods

Sampling, pretreatment and enumeration of bacterial populations. Raw and treated sewage and sludge from a biological treatment plant located in the north east of Spain were sampled. The plant daily treats 2,000 m^3 from a population of 5,000 habitants. Hydraulic retention time of the sewage was 2.2 days and the treatment method was by aerated sludges. Two kinds of sludge samples were taken: liquid sludge from the recycling sludge channel, with a dry weight residue of 0.4–0.6 g/100 ml, and dewatered sludge obtained at the end of the process, with 14–15 g/100 ml of dry weight residue. Samples were collected and stored at 4°C following standard protocols (Anonymous, 1994). Dewatered sludge samples were homogenized by magnetic agitation during 30 min in a solution of Ringer 1/4 in proportion 1 : 10 w/v, while liquid sludge samples were homogenized in the same way without the addition of Ringer.

The enumeration of fecal coliforms and enterococci was performed by membrane filtration (Anonymous, 1997a, b) on 0.45 μm of pore size membranes (Millipore, Molsheim, France). Filtrated samples were cultured on m-FC Agar (mFCA) plates (Difco, Detroit, USA) at 44.5°C for 24 h to enumerate fecal coliforms. Counts of blue colonies were done at 24 h (Grabow, 1990). In addition, another 3 sets of membranes with filtrated samples were pre-incubated on Brain Heart Infusion Agar (BHIA) (Difco) at 37°C for no more than 2 h for the recovery of stressed enterococci (Anonymous, 1998). Membranes were then transferred onto m-*Enterococcus* Agar plates (MEA) (Difco), MEA with 8 mg L^{-1} of erythromycin (Sigma-Aldrich, Saint Quentin Fallveir, France) and MEA with 8 mg L^{-1} of vancomycin (Sigma-Aldrich), for the enumeration of total enterococci and, enterococci resistant to erythromycin and to vancomycin respectively as previously described (Blanch et al., 2003). Plates were incubated at 37°C for 48 h. Then they were transferred to Bile Esculine Agar (BEA) (Difco) for 1 h at 44°C to confirm the enterococci colonies on the basis of the hydrolysis of esculine (Manero and Blanch, 1999). Additionally, because of the low concentration of VRE strains that was assumed, enrichment in Enterococcosel™ broth (Becton Dickinson, Cockeysville, MD, USA) was performed in order to isolate resistant strains for the diversity index calculations. Aliquots of 10 ml of treated sewage samples were inoculated to double concen-

trated Enterococcossel™ broth containing 16 mg L^{-1} of vancomycin. To isolate strains, aliquots of 10 µl from tubes showing growth were seeded onto MEA plates containing 8 mg L^{-1} of vancomycin. Finally, pure cultures of these strains were obtained and confirmed by hydrolysis of esculine on BEA as described above.

Spores of SRB were enumerated by thermic-shock of samples (homogenization 1 : 10 in the case of sludges) at 80°C for 10 min (Handford, 1974). Later, ten-fold dilutions were made in Ringer 1/4, and 1 ml of each dilution was inoculated in 50 ml of liquid Sulphite Polymyxin Sulfadiazine (SPS) Agar (Scharlau, Barcelona, Spain). Inoculated tubes were shaken to homogenize the solution and allowed the media to solidify. These tubes were then incubated at 44°C for 24 h.

Biochemical fingerprinting. A maximum of 24 colonies of each bacterial group (fecal coliforms and enterococci) were randomly isolated from plates showing between 30 and 100 colonies for each sample. These colonies represent the fecal coliforms and enterococci associated with each sample (Bianchi and Bianchi, 1982). Overnight cultures of enterococci and fecal coliforms isolates on BHIA were prepared at 37°C and 44.5°C respectively for biochemical fingerprinting with the Phene Plate System (PhP-Plate Microplates Techniques AB, Stockholm, Sweden). Cell suspensions were prepared by harvesting these cultures in a suspending medium: 0.2% w/v proteose peptone (Difco), 0.05% w/v yeast extract (Scharlau) 0.5% w/v NaCl and 0.011% w/v bromothymol blue (Merck, Darmstadt, Germany) for enterococci, and 0.1% w/v proteose peptone and 0.011% w/v bromothymol blue for fecal coliforms. These cell suspensions were performed in the first well of each row of the PhP-RE and PhP-RF microplates (PhP-Plate Microplates Techniques AB) respectively, by picking up and resuspending a loopful of culture in 300 µl of the suspending medium. Aliquots (25 µl) of the bacterial suspension of this well were transferred to the other wells in the same row, following the manufacturer's instructions and as previously described (Kühn and Möllby, 1993).

The PhP-RF and PhP-RE plates consist of 96-well microplates containing dehydrated reagents, which have been selected to provide a high level of discrimination of populations within enterococci or fecal coliforms respectively (Kühn et al., 1991, 2000). The biochemical fingerprinting bases of these microplates has been described previously (Kühn, 1985). Inoculated PhP-RF and PhP-RE microplates were incubated at 37°C. Growth in wells was measured by using the iEMS Reader MF (Labsystems Helsinki, Finland) at 620 nm. Three readings were performed at 16 h, 40 h and 64 h for enterococci, and at 7 h, 24 h and 48 h for fecal coliforms. The biochemical profiles were calculated for each isolate as previously described (Kühn et al., 1991) and using the software PhpWin® (PhP-Plate Microplates Techniques AB).

Indexes of population diversity and similarity. The Simpson's diversity index (*Di*) was used to calculate the diversity of the bacterial populations in each studied group while the similarity between populations was calculated by the coefficient of population similarity (*Sp*) (Kühn et al., 1991). Then, the diversity indexes were calculated considering all the isolates of fecal coliform and enterococci for each kind of sample. The comparison of the populations of these bacteria between different kinds of samples was evaluated using the unweighted-pair groups method analysis (UPGMA) with average linkage, clustering analyses and calculations of *Sp* coefficients. The reading, calculations of indexes, correlation coefficients and clustering analyses were also performed using the PhpWin® software (PhP-Plate Microplates Techniques) as previously described (Kühn et al., 1991).

Identification of clusters and classification of isolates. Different clonal populations were determined by clustering analyses on the basis of their biochemical fingerprinting (PhP-profiles). Clusters were constituted by isolates which presented a correlation coefficient of PhP-profiles higher than 0.975. FC and ENT isolates were identified by comparing their biochemical PhP-profiles obtained in the present study with those which were identified in previous studies (Blanch et al., 2003; Vilanova et al., 2004). Then, species identification of the FC representative isolates was performed using the API 20E gallery following the manufacturer's instructions and database profiling (bioMérieux, La Balme, France). Enterococci representative isolates were identified using standard methods and the *Enterococcus* matrix described elsewhere (Manero and Blanch, 1999) and the Bacterial Identifier software (Blackwell Science Publishers, Ltd., Oxford, UK). Moreover, enterococci clusters belonging to phylogenetically related species (Behr et al., 2000) such as *Enterococcus hirae* and *Enterococcus durans* (H-D group) and *Enterococcus casseliflavus*, *Enterococcus*

Table 1. Enumeration and diversity of the analyzed bacterial populations.

	CI	FC		ENT		ERE		VRE
	Load	Load	*Di*	Load	*Di*	Load	*Di*	Load
RS	5.55 (±0.31)	7.20 (±0.31)	0.988	5.99 (±0.31)	0.944	5.03 (±0.48)	0.927	1.89 (±0.59)
TS	3.61 (±0.45)	4.57 (±0.29)	0.991	3.37 (±0.46)	0.963	2.26 (±0.55)	0.908	<1
LS	6.08 (±0.10)	6.19 (±0.16)	0.986	5.79 (±0.20)	0.918	4.50 (±0.02)	n.d.	2.15 (±0.15)
DS	7.59 (±0.08)	7.55 (±0.29)	0.991	7.16 (±0.15)	0.961	6.27 (±0.10)	n.d.	2.69 (±0.98)

Load: average of log (CFU×100 ml^{-1}) for sewage and liquid sewage or log (CFU×100 g^{-1}) for dewatered sludge; RS: raw sewage; TS: treated sewage; LS: liquid sludge; DS: dewatered sludge; *Di*: population diversity. Values of standard deviation for colony counts are indicated in brackets. CI: spores of sulphite-reducing anaerobes (clostridia); FC: fecal coliforms; ENT: enterococci; ERE: enterococci resistant to 8 mg L^{-1} of erythromycin; VRE: enterococci resistant to 8 mg L^{-1} of vancomycin; n.d.: not determined.

gallinarum and *Enterococcus flavescens* (C-G-F group) were considered as a unique group (Blanch et al., 2003). Those FC and ENT isolates not identified were grouped in "Others." The proportions of the main bacterial species or genus were then calculated for each kind of sample and bacterial group as previously described (Vilanova et al., 2004).

Results

Enumeration of bacterial populations

A total of six samples was taken from each kind of sewage and sludge. The treatment plant showed a reduction of around 2.5 log units of colony counts for fecal coliform and enterococcal populations and 2 log units of colony counts for sulphite-reducing bacteria. Liquid sludge showed at least 1 log unit counts lower than dewatered sludge, and this presented the highest concentrations for all the studied populations (Table 1). The counts for erythromycin-resistant enterococci (ERE) were always around 1 : 10 with respect to the total counts of enterococci. Vancomycin-resistant enterococci (VRE) were detected at very low proportion in raw sewage and liquid and dewatered sludge, being around 1 : 50,000 vancomycin-resistant counts in respect to the count of total enterococci (Table 1). However, VRE strains were also isolated from treated sewage by the treatment of enrichment described above.

Diversity index and population similarities between sampling sites

A total of 423 fecal coliforms and 544 enterococci strains were isolated and phenotyped. High values of diversity indexes (>0.89 in all cases) were found for both bacterial groups in all type of samples (Table 1). However, these indexes were always slightly higher for fecal coliforms. The diversity indexes for VRE samples from sludge were not determined because the total number of isolates obtained from these samples was not sufficient for calculation. The population similarity indexes (*Sp*) between the different kind of sample were high for both bacterial groups (Fig. 1), though enterococci populations showed higher similarity (*Sp* values between 0.32 and 0.47 vs. 0.15 and 0.25 for fecal coliform populations). However liquid and dewatered sludge on one side and treated and raw sewage on another side were more similar among them for any studied bacterial group.

Proportions of distinct species within fecal coliforms and enterococci populations

The most frequent fecal coliforms were isolates belonging to *E. coli* biochemical fingerprinting profiles, followed by *Citrobacter* spp. in any kind of sample (Table 2). However, many of the representative isolates, which belonged to Php-profiles with a low number of isolates, were not identified. *Ent. faecalis* and *Ent. faecium* were the most abundant enterococci species in all studied samples, representing together always most of the 50% of all samples (Table 2). Other *Enterococcus* species in lower proportion were *Ent. hirae* and *Ent. durans* (H-D group) followed by the group of *Ent. casseliflaus*, *Ent. gallinarum* and *Ent. flavescens* (C-G-F group). Other enterococcal populations which were not identified ranged from 12.9% (raw sewage) to 45.3% (dewatered sludge).

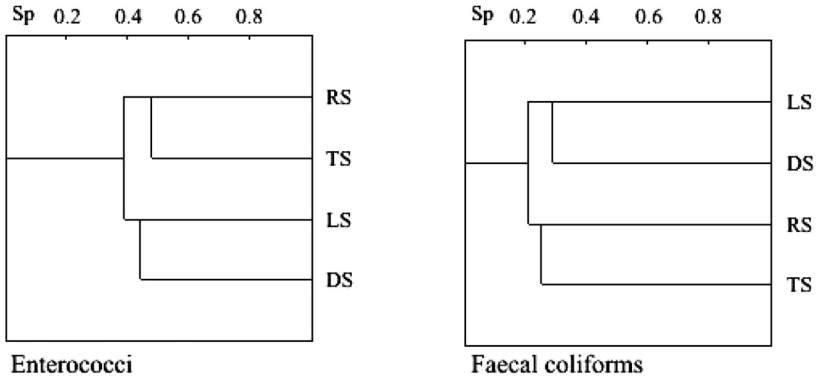

Fig. 1. Dendrograms of the clustering analyses based on the similarity of population index (*Sp*) between the different studied samples.

RS: raw sewage; TS: treated sewage; LS: liquid sludge; DS: dewatered sludge.

Table 2. Percentage of the most abundant genus of fecal coliforms and *Enterococcus* spp. in the different kinds of samples.

	Fecal coliforms					Enterococci				
	EC	CIT	KLEB	ENTB	Others	EFL	EFM	H-D	C-G-F	Others
RS	29.1	7.4	0.3	0.6	62.6	36.5	38.3	7.6	4.7	12.9
TS	18.2	7.8	0.6	2.6	70.8	25.9	38.0	11.6	3.5	21.0
LS	14.6	10.4	0.0	4.2	70.8	29.8	29.2	25.0	2.1	25.0
DS	24.0	1.3	0.0	3.8	70.7	10.9	32.8	9.4	0.0	45.3

RS: raw sewage; TS: treated sewage; LS: liquid sludge; DS: dewatered sludge; EC: *Escherichia coli*; CIT: *Citrobacter* spp.; KLEB: *Klebsiella* spp.; ENTB: *Enterobacter* spp.; EFL: *Enterococcus faecalis*; EFM: *Enterococcus faecium*; H-D group: *Enterococcus hirae* and *Ent. durans* group; C-G-F group: *Enterococcus casseliflavus*, *Ent. gallinarum* and *Ent. flavescens* group. Others correspond to non-identified isolates.

Discussion

While fecal coliform predominated among the studied populations in raw and treated sewage, in both kind of sludge the three studied bacterial groups tended to occur in similar proportions. This situation could be related to the inclusion of bacteria into sludge particles as other authors have suggested (Stenstrom and Carlander, 2001). The increase of concentration in dewatered sludge is directly proportional to the increase of dry weight. These results are partially in agreement with those of other authors (Chauret et al., 1999) who found a similar situation at least for raw sewage, primary effluent and sludge. The proportions between the studied bacteria populations and their reduction between raw and treated sewage are in agreement with previous studies (Hill and Sobsey, 1998; Payment et al., 2001; Vilanova et al., 2004). Spores of SRB were found in higher proportion in respect to the other bacterial groups in treated sewage than in raw sewage. These differences are explained by a higher die-off for FC and ENT than for SRB (Fujioka et al., 1981; González et al., 1990; Mocé-Llivina et al., 2003). Though changes in the ENT/FC ratio could be expected in treated sewage, a similar die-off in treated sewage for FC and ENT was detected and consequently no changes were observed for this ratio. Moreover, the obtained results showed a similar composition and structure of bacterial populations between raw and treated sewage that suggest no differential die-off for those studied bacterial groups (FC and ENT). Finally, the relative concentration of spores of SRB in liquid sludge increased in respect to raw sewage, whereas it decreased in the case of the other bacterial groups. This could be explained by the aeration process, which could induce the sporulation of SRB.

While in treated sewage both fecal coliform and

enterococci populations presented a similar die-off, enterococci populations accumulate in a higher proportion than do fecal coliforms in sludge. This could be explained if one of the principal factors of elimination into the sludge is the predation by ciliated protozoa, because as was reported by other authors (González et al., 1990) this predation selectively affects gram negative bacteria, such as fecal coliforms.

ERE strains accounted for 10% of the total enterococci in all the analyzed samples. VRE strains were found in very low concentration (around 0.01% of total enterococci) but persisted in the treated sewage and also in sludge. However, there was no evidence of any differential factor that promoted higher persistence or reduction of resistant strains. This finding contrasts with that reported elsewhere in which a higher proportion of resistant strains of fecal coliforms (Mezrioui and Baleux, 1994) or total coliforms (Andersen, 1993) were observed in treated sewage or sludge. Though in a low proportion, bacterial populations carrying antibiotic resistance can persist in the sludge as well as in the treated water. This aspect should be considered when sludge is disposed of or used for diverse soil applications.

In all studied samples, *Ent. faecium* and *Ent. faecalis* were the predominant enterococci species while *E. coli* and in a second percentage *Citrobacter* spp. were the predominant fecal coliforms. These results are in agreement with previous studies (Brown and Tracey, 1975; Hill and Sobsey, 1998; Laukova and Juris, 1997; McLellan et al., 2001; Sinton and Donnison, 1994; Svec and Sedlacek, 1999; Vilanova et al., 2004). *E. coli* was the main fecal coliform in any kind of the studied samples. However, *Enterobacter* spp. were found in higher proportion than *Citrobacter* spp. in dewatered sludge. *Ent. faecium* is the most abundant *Enterococcus* spp. in any sample being clearly the main group in dewatered sludge though a higher proportion of enterococci were not identified in this kind of sample. High diversity indexes for fecal coliforms and enterococci populations were found in all the samples. The high similarity population indexes support that FC and ENT have similar composition and structure of populations. This is not modified by the sewage treatment or sludge dewatering process. Consequently, the change of the proportions of FC and ENT between sludge and the water samples were not translated in changes of species proportions inside any studied bacterial group. Though both bacterial groups showed high similarity of populations in any case, liquid and dewatered sludge were more related to each other than to the two kinds of analyzed sewage. The high similarity of clonal populations between raw and treated sewage obtained in this study is also in agreement with previous studies that compare several treatment plants (Vilanova et al., 2004).

In conclusion, the obtained results suggest the main reduction of the studied indicators in an aerobic treatment sewage plant with a short retention time is their inclusion in sludge, where FC have the highest reduction in respect to the other analyzed indicators. The composition and structure of FC and ENT populations are similar among sewage and sludge. It is confirmed by the persistence of VRE and ERE populations at the same proportions not only in treated sewage but also in liquid and dewatered sludge. The persistence of antibiotic-resistant strains in sludge should not be underrated in the disposal or reutilization of sludge.

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