UNIVERSITAT DE BARCELONA

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Facultat de Biologia

Diversitat de les poblacions de coliforms fecals i d'enterococs a les aigües residuals i anàlisi de les modificacions en la composició i l'estructura poblacional a les plantes depuradores.

Xavier Vilanova Solà

Tesi doctoral
Barcelona, 2005

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Programa de doctorat: Microbiologia ambiental i biotecnologia (1999-2001)

Vist-i-plau del Director de la tesi,

Dr. Anicet Blanch Gisbert.

Memòria presentada per
Xavier Vilanova Solà,
Per optar al grau de Doctor per la
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Els homes de la Terra van venir a Mart. Van venir-hi perquè tenien por o perquè no en tenien pas, perquè eren feliços o perquè no ho eren gens, perquè se sentien com els Pelegrins o perquè no s'hi sentien mica. Cada home tenia el seu motiu (...)

Des de cartells a quatre tintes, un dit del govern assenyalava tothom, a totes les ciutats: AL CEL HI HA FEINA PER VÓS:VENIU A MART!

Ray Bradbury
Les cròniques marcianes.

Agraïments

Preàmbul

Avui podria ser una tarda qualsevol i podria escriure una llista de noms com qui es prepara per anar a comprar. Es curiós com les casualitats van conformant la nostra vida; com aquell dia en que venia a veure el Marc i em vaig trobar al Jordi Escoda. Em va comentar que al departament de Microbiologia buscaven una persona per fer tasques de mostreig. Es curiós també que aquell fos el darrer dia de presentar les sol·licituds i que una noia molt amable, em presentés a qui acabaria sent el meu director de tesi, l'Anicet Blanch. Les casualitats continuen i és que en aquell temps estava fent la Prestació Social Substitutòria al Cim d'Àligues, on la Lourdes, el Rafa, la Itziar, els Jordis, el Francesc i companyia em van posar les coses molt fàcils quan els vaig explicar que tenia la possibilitat de treballar al Departament de Microbiologia de la U.B. I quan vaig començar a treballar l'Albert, gran aficionat a la ornitologia (curiós oi?) va ser qui m'ensenyà el funcionament del Departament. Aleshores estàvem apretats al laboratori 38 amb la Marta Cerdà, la Yolanda, la Laura, i la Melanie. Al costat, a *la Sibèria*, teníem la Lis, la Sandra, la Rosa, la Sònia la Teresa i l'Olga i de vegades, quan podíem coincidir, esmorzàvem plegats. També hi treballaven algunes persones amb qui no vaig tenir ocasió de tractar gaire, com el Carles, la Cristina Madrid, el Ricard, el Toni i el José Maria. Més lluny (al principi el Departament em semblava molt gran) hi havia la Idoia (la noia que m'havia presentat a l'Anicet) la Sílvia i el Marc, i una colla de gent que aniria coneixent mica en mica. Estirant del fil, recordo també les primeres lectures de tesi a les que vaig assistir (Xavi Rubires, la Montse i l'Anna Puig, la Maite, el Jordi Sabaté, la Teresa Guindulain...). Pensant en aquells inicis inevitablement apareix el Jordi Dellundé, que em va ensenyar les tècniques i els punts de mostreig del grup, i amb qui recordo els desplaçaments amb cotxe i les converses sobre bàsquet i pesca. Unes coses porten a les altres i com que no es pot tenir tot, en aquella època vaig haver de deixar el bàsquet després de 15 anys

federat. L'altre cosa que m'evoca el Jordi és la *Leonera* i "como la cabeza al sombrero" al Javi Méndez i la Núria Contreres. No va trigar gaire a arribar l'Ana Emilia, de qui havia sentit parlar molt: de seguida vaig comprendre per què. En aquestes alçades de la pel·lícula tampoc em puc estar de recordar els sopars i les gresques que fèiem i la sensació de "família" que tenia, ja coneixia també al Toni Navarrete, el Xavi Huete, la Marga, la Núria Prim, la Maria, l'Ina, el Lluís, l'Ester, la Cristina, la Glòria la Susanna Guix, el Santi, la Rosario i l'Alberto.

El canvi generacional, un desconcertant congrés a Granada i el trasllat de mig departament cap a l'edifici nou van marcar un abans i un després. A partir d'aquí els records se m'agrupen a l'entorn d'esdeveniments. Recordo el meu segon dinar de Nadal amb les noves incorporacions del grup (Cristina, Joan Lluís, Pili, Gonzalo i Meritxell) i el tercer (Michel, Eli, Xavi Bonjoch, ...).També penso en la gent que va marxant amb un futur engrescador (L'Olga, les Sandres, la Sonia Pina...) moments dolorosos i de solitud pel Xavi Abad, la Núria Queralt, la Meritxell, la Lis... però també d'alegria pel naixement de la Fransesca, la Maël, la Núria; el casament de la Núria i el Xavi, el Gonzalo i la Idoia... Una tesi dóna per tant... També dóna per conèixer gent d'altres països: la Melanie i el Thomas (França), l'Ana Emilia, la Mari Luz i l'Andrey (Colòmbia), el Cristof i l'Alex, el Cyril, la Karine (França), la Zaira (Mèxic), l'Hugo (Portugal), l'Ayalke (Etiòpia), el Jorge (Argentina), la Heidi (Holanda)...

Darrerament i gràcies a les ganes de fer coses, amb i per la gent, del Marc, el Quim i la Marta les distàncies entre els dos edificis s'escurcen i l'Oscar, el Cristian, la Laura i la Sònia em recorden altres temps...). I què dir del meus companys de laboratori, el Xavi Bonjoch, la Cristina García i l'Eli, que juntament amb el Michel, l'Andrey, l'altra Eli, la Susana, la Cristina, l'Ester, l'Anna, la Marta, el Carles, el Néstor i la Ceci donen "vidilla" a l'edifici vell.

Ja em comença a costar recordar cares i noms, i degut a les meves noves ocupacions a l'Aquarium i a l'Ajuntament de Caldes, és possible que els extrems temporals d'aquest "retrat de família" puguin distorsionar-se una mica, per això la gent que no hi sou amb noms, també m'alegro d'haver compartit amb vosaltres

aquest espai que ha estat per a mi com una "segona casa" durant molt de temps. A tots i totes, moltes gràcies.

L'amistat.

És evident (llei de vida podríem dir-ne) que d'aquí un temps el record d'aquest 5 anys s'anirà difuminant. Les persones que anem marxant farem visites cada cop més esporàdiques, i ens n'adonarem que els cicles de la vida es van repetint també dintre el Departament. Recordarem amb nostàlgia molts moments viscuts i noves coneixences ocuparan mica en mica el lloc deixat per alguns de vosaltres. Espero que després de riure i plorar junts, de viure amb tanta intensitat entre dies de rutina, em trobi almenys amb tu, amic, amb tu, amiga, i que de sobte el cor ens bategui una mica més de pressa, que els nostres ulls es mirin i que tornin aquells somriures tan especials que només tu i jo coneixem.

La Tesi doctoral

No oblidem que la tesi doctoral és un aprenentatge, no només de la vida, sinó també de la ciència i el seu mètode; una cosa sense l'altre no tenen cap sentit. I evidentment també s'aprenen a utilitzar diverses eines i a moure's en diferents ambients. Des dels idiomes, passant per tècniques d'ofimàtica, presentacions de treballs, reunions amb grups d'investigadors d'altres països i fins a gestions, amb i per diferents administracions públiques i privades, són exemples d'oportunitats d'aprenentatge que una tesi doctoral ofereix. És per tot això que haig d'agrair a l'**Anicet Blanch** la possibilitat que em va brindar de guiar-me en aquest camí, tant en la part humana, com en la científica. Sempre has estat disposat a escoltar-me, a corregir-me i a ajudar-me. Gràcies també per permetre'm la compaginació dels treballs de tesi amb els treballs a l'Aquàrium de Barcelona, i a l'Ajuntament de Caldes. Sense els teus ànims i el teu suport no m'hauria estat possible. Gràcies també al Francisco Lucena i al Joan Jofre per les valuoses aportacions dels vostres coneixements sobre microbiologia d'aigües així com per l'interès i el suport que

m'heu demostrat envers el compromís i la responsabilitat que vaig assumir amb el meu poble.

Gràcies Temi per mostrar-me un punt de vista nou per a mi de la ecologia microbiana: el concepte de "mort" aplicat als bacteris. Gràcies a l'Umbert que em va apropar una mica al món dels protozous i al Narcís Prat per les dades hidrogàfiques facilitades. A la Rosina, la Rosa, a l'Albert Bosch, l'Antonio Juárez i a tots aquells professors i professores que sempre esteu disposats a donar-nos un cop de mà, a compartir amb nosaltres els vostres coneixements. Gràcies de nou als companys i companyes que m'heu ajudat a aprendre coses sobre els diferents microorganismes amb els que heu treballat: la seva ecologia, els seus gens, el seu metabolisme, etc. És amb totes aquestes peces que és possible gaudir d'una tesi.

Les altres ocupacions...

A tota aquella gent de l'Ajuntament de Caldes, tècnics i polítics que heu sabut suplir les meves limitacions amb la vostra col·laboració. Gràcies també al Marc Ordeix, a l'Àngel Valeriano, el Sr. Padilla, al Sr. José i a la resta dels responsables de manteniment de les EDARs i hospitals respectivament, al Josep i el Dr. Solà Peiró, al Josep i a la veterinaria, l'Anna Romagosa. I a vosaltres Coral, Susanna, Marta, Xavi, Lucía, Patrici i companyia, que m'heu ensenyat molt sobre els peixos.

Familiars i amics

Tot i la brevetat d'aquest apartat, la llista de noms i les coses que m'heu donat per arribar aquí seria inacabable. Més que agrair-vos-ho voldria demanar-vos disculpes pel temps que no us he pogut dedicar. Espero que sabreu perdonar-me.

Als meus pares

Ai parella, que n'heu hagut d'aguantar de cares llargues, mals humors i feines extres (...) i sense obtenir-ne cap recompensa. I és que els pares sempre us emporteu la pitjor part. Encara que el paràgraf d'una tesi sigui molt poca cosa us vull agrair tot el que heu fet per mi, la paciència que heu tingut i l'educació que m'heu donat. Sense vosaltres és del tot segur que res d'això hauria estat possible. Moltes gràcies.

A la Mari

Segur que tu ets la persona amb qui més temps he passat els darrers 9 anys de la meva vida. Per tant també has viscut aquesta tesi com una cosa teva. Però després del que hem compartit una tesi doctoral es fa tan poca cosa (...)

Estiu de 2003.

Índex

INTRODUCCIÓ.

1– Diversitat poblacional dels indicadors bacterians.

La presència de contaminació fecal en les aigües continentals i marines és un fenomen freqüent tal i com constaten diferents estudis. Aquesta presència arriba a concentracions elevades en les aigües residuals urbanes que es tracten a les depuradores. Un cop tractades, les aigües residuals són retornades als sistemes hidrològics, ja sigui als rius, ja sigui al mar, amb una càrrega de bacteris fecals menor però encara present. El tipus de depuradores utilitzades determinarà canvis qualitatius i quantitatius a les poblacions de bacteris fecals de l'aigua. El tractament d'aigües residuals és un dels processos biotecnològics de major escala a nivell mundial i entre els comercialment més importants. No obstant, i malgrat el desenvolupament en els darrers anys de tècniques moleculars, les interaccions entre les poblacions microbianes que intervenen activa o passivament al llarg d'aquests són encara poc conegudes. Per exemple, la distribució de les espècies dels indicadors bacterians més utilitzats per controlar la càrrega fecal al llarg dels tractaments a les depuradores (coliforms fecals i enterococs) ha estat molt poc estudiada. S'ha observat que les espècies dominants d'enterococs en aigües residuals són *Enterococcus faecium* i *Enterococcus faecalis*. (**Sinton i Donnison 1994; Laukova i Juris 1997; Svec i Sedlacek 1999).** En el cas dels coliforms fecals, s'ha descrit que una elevada proporció d'aquests indicadors en aigües residuals de depuradora pertanyen a l'espècie *Escherichia coli* **(Hill i Sobsey 1998)**. Aquesta elevada proporció d'*E. coli* dintre els coliforms fecals ja fou detectada anteriorment per **Jouenne i col.** (**1985**) a l'analitzar aigües de diferents orígens (residuals, superficials, de consum i subterrànies). En aquest cas els altres gèneres detectats, tot i que en unes proporcions molt baixes, foren *Klebsiella*, *Citrobacter* i *Enterobacter*.

La presència d'uns o altres grups bacterians, ja sigui a nivell de gèneres, espècies o fenotips, així com les proporcions d'aquests, podria ser utilitzat com a indicador del bon funcionament del procés de tractament, de l'origen de les possibles contaminacions, o la procedència d'una aigua residual (**Sinton i Finlay 1998**). Caldria però un patró de referència, que si bé podríem trobar-lo a la bibliografia amb un cert grau de consens a

nivell de proporcions d'alguns indicadors en una aigua residual urbana estàndard (**Biton 1994, Lucena i col. 2004**) resulta més complex trobar-lo una vegada aquesta aigua passa per diferents sistemes de tractament, o si es vol analitzar a nivells de diversitat d'espècies o perfils bioquímics dintre d'aquestes espècies.

Tot i que a mitjans del segle XX la biodiversitat comença a plantejar-se com a eina per interpretar processos ecològics com la successió, la competència o la productivitat d'una comunitat, no és fins a finals dels anys 60 i principis dels 70 que aquest concepte es trasllada al camp de la microbiologia (**Hariston i col. 1968; Swift 1974**). No obstant, mentre que en el cas de l'estudi d'organismes macroscòpics hi ha molta bibliografia per a desenvolupar estratègies de mostreig i anàlisi, quan es tracta de microorganismes la tasca resulta més complicada. En funció dels paràmetres que s'utilitzin per a calcular la diversitat, s'obtindran resultats diferents per un mateix ecosistema. Sovint, els factors crítics alhora de definir una estratègia de mostreig són les tècniques per a la detecció, la classificació, la quantificació i la determinació de l'estat vital de les cèl·lules detectades. Algunes tècniques moleculars permeten detectar la presència de fragments d'àcids nucleics, proteïnes, lípids o sucres específics de determinats grups bacterians, ja sigui a nivell de família, gènere, espècie o soca; però sovint la seva detecció no permet conèixer el seu estat fisiològic, o la concentració en la que són presents a la mostra analitzada. A més l'aplicació d'algunes d'aquestes tècniques moleculars no és factible en mostres d'aigua molt tèrbola, amb elevada càrrega microbiana i especialment si aquesta presenta una elevada diversitat. Darrerament tècniques com la citometria de flux estan obrint noves perspectives (**Forster i col. 2003**) però encara sembla difícil que es puguin aplicar de forma rutinària i per qualsevol tipus de mostra, ja que demanen un gran esforç per la seva posada en funcionament així com un elevat cost econòmic. També són factors crítics el definir les poblacions sobre les quals es faran els estudis de diversitat (no és el mateix estudiar la diversitat d'una espècie, que la d'un gènere o una família) o la representativitat de la mostra a analitzar respecte l'ecosistema objecte d'estudi (**Morris i col. 2002**).

Els estudis de la diversitat en el cas de les poblacions dels bacteris fecals en aigües residuals o contaminades resulten doncs complexos i costosos, pel tipus de mostres, la concentració bacteriana elevada, la manca d'un patró estàndard sobre el que

comparar i la caracterització fenotípica i/o genotípica dels clons poblacionals. Tot i així, existeixen estudis que utilitzant diferents tècniques analitzen la diversitat de diferents grups bacterians en diferents tipus de mostres. **Kühn i col.** (**1997**) analitzaren els clons poblacionals de coliforms en aigües residuals industrials i detectaren diferències entre les poblacions de coliforms de les aigües residuals de 3 indústries papereres i les de les aigües del riu que en rebia els abocaments, al llarg de diferents trams. Aquesta diferència es posava de manifest, entre d'altres coses, per la baixada de la diversitat de les poblacions analitzades en els punts d'abocament (amb un predomini del gènere *Klebsiella*) i la recuperació d'aquesta diversitat uns kilòmetres riu avall. També **Gauthier i Archibald (2001**) analitzaren la diversitat de poblacions de coliforms en aigües residuals d'una indústria paperera, i observaren que alguns clons poblacionals de l'espècie *Klebsiella* presumiblement es replicaven a l'aigua. **McLellan i col. (2001)** descrivien, en aigües recreacionals, diversos clons poblacionals pertanyents als gèneres *Klebsiella*, *Citrobacter* i *Enterobacter* que presumiblement replicarien en aquest entorn, cosa que es reflectia amb una baixada de la diversitat. Aquests treballs assenyalen que l'estudi a nivell de clons poblacionals d'alguns indicadors podria proporcionar informació sobre les diverses fonts de la contaminació de les aigües i els efectes dels diferents tractaments rebuts per les aigües en l'estructura d'aquestes poblacions.

Els indicadors escollits en aquest treball (enterococs i coliforms) són, juntament amb *Bacteroides*, *Bifidobacterium* i *Eubacterium*, els bacteris més abundants de la microbiota intestinal humana (**Mitsuoka, 1984**). Tot i que a l'intestí la concentració d'enterococs i coliforms és d'entre 1 i 3 logaritmes inferiors a les tres poblacions anaeròbiques citades anteriorment, els valors detectats en aigües residuals estan en el mateix rang o lleugerament per damunt dels coliforms fecals (**Bonjoch i col. 2005**). Aquestes diferents proporcions en les aigües residuals respecte a l'intestí poden explicar-se pel caràcter anaeròbic d'algunes poblacions intestinals que els fa tenir una baixa persistència en el medi ambient. Pel que fa a les espores de clostridis sulfit-reductors, malgrat estar molt qüestionades com a indicador pel seu possible origen tel·lúric, l'elevat grau de supervivència que presenten en l'ambient aporta un valor de referència que resulta d'interès en aquest treball. A més a més, serveix com a un marcador més per comparar i establir proporcions relatives amb les altres poblacions analitzades.

La comparació de les poblacions de coliforms fecals i enterococos a l'entrada i a la sortida de diferents depuradores podria posar de manifest l'eliminació selectiva de determinades soques, algunes d'interès clínic, com poden ser aquelles que presenten resistència a antibiòtics. Aquestes resistències podrien correspondre a unes soques determinades o distribuir-se homogèniament dintre les poblacions bacterianes. En aquest sentit els estudis realitzats per **Mezrioui i Belaux (1994)** detectaven un increment de la proporció de coliforms fecals resistents a antibiòtics en l'aigua tractada d'una depuradora, i per tant, apuntaven a l'existència d'una eliminació selectiva de determinades poblacions pels processos de tractament d'aigües. Cal disposar però d'un nombre d'estudis més elevat per a poder confirmar aquest tipus de fenòmens i comprendre millor els efectes selectius que podrien tenir les plantes de tractament d'aigües residuals. L'esforç invertit per obtenir les dades suficients per a donar consistència als resultats pot ser doncs, de vegades, un factor limitant. Però l'aparició de tècniques de fenotipatge bioquímic miniatruritzades i mecanitzades, com les plaques BIOLOG (Biolog Inc., Hayward, CA, USA) o el Phene-Plate System (PhP-Plate Microplates Techniques AB, Sweden) ha facilitat l'estudi de la diversitat i, en algun cas, la identificació taxonòmica. Les microplaques del Phene-Plate System permeten analitzar una gran quantitat de soques de manera ràpida i senzilla, cosa que proporciona una bona representativitat estadística de les mostres. A més a més, el programa informàtic desenvolupat pels fabricants d'aquest sistema permet un tractament estadístic de les dades acumulades, ràpid i de fàcil interpretació, així com el càlcul de la diversitat i la comparació tant entre dos perfils bioquímics determinats com entre poblacions senceres. Aquesta tècnica és de gran utilitat també per proporcionar soques ambientals "contextualitzades" dintre de les poblacions estudiades (proporció que representa, persistència relativa, origen, etc.) per a posteriors treballs de biologia molecular (**Kühn i col. 2000; Hasman i Arestrup 2002**). Els detalls de la tècnica del Phene-Plate System es poden consultar en els diferents articles que composen aquesta tesi, i més àmpliament al següent apartat d'aquesta introducció.

Atenent al limitat coneixement de la diversitat de les poblacions microbianes en les aigües residuals i l'avaluació de l'eliminació selectiva o no d'alguns clons poblacionals, en els estudis realitzats en aquests treballs es planteja aportar noves dades

per a respondre aquests aspectes. Per tal de realitzar els estudis de l'estructura i la composició de les poblacions bacterianes s'han utilitzat dues eines metodològiques: un sistema de fenotipatge bioquímic en microplaca (Phene-Plate System) i l'enumeració de poblacions d'enterococs que presenten resistència a l'eritromicina o a la vancomicina. En els següents apartats es descriuen en detall aquests instruments d'anàlisi utilitzats.

2– El Phene Plate System aplicat a l'anàlisi en aigües residuals.

El Phene-Plate System és un mètode de fenotipatge bioquímic que permet processar un elevat nombre de soques bacterianes, basant-se en un sistema miniaturitzat en microplaques de 96 pous. S'han desenvolupat diferents microplaques per a poder fenotipar diferents grups bacterians. Les plaques PhP-RE i PhP-RF (PhP-Plate Microplates Techniques AB, Sweden) consten de 8 pous d'inoculació i 88 amb reactius deshidratats que han estat seleccionats perquè permeten una alta discriminació entre els coliforms fecals i els enterococs respectivament. En la Taula 1 es relacionen els substrats que constitueixen cadascuna d'aquestes plaques de 8 files x 12 columnes. Els fonaments del fenotipat bioquímic mitjançant aquestes plaques van ser descrits per **Kühn** (**1985**) i s'expliquen en els següents subapartats.

Taula 1 Substrats continguts en les plaques PhP-RE i PhP-RF

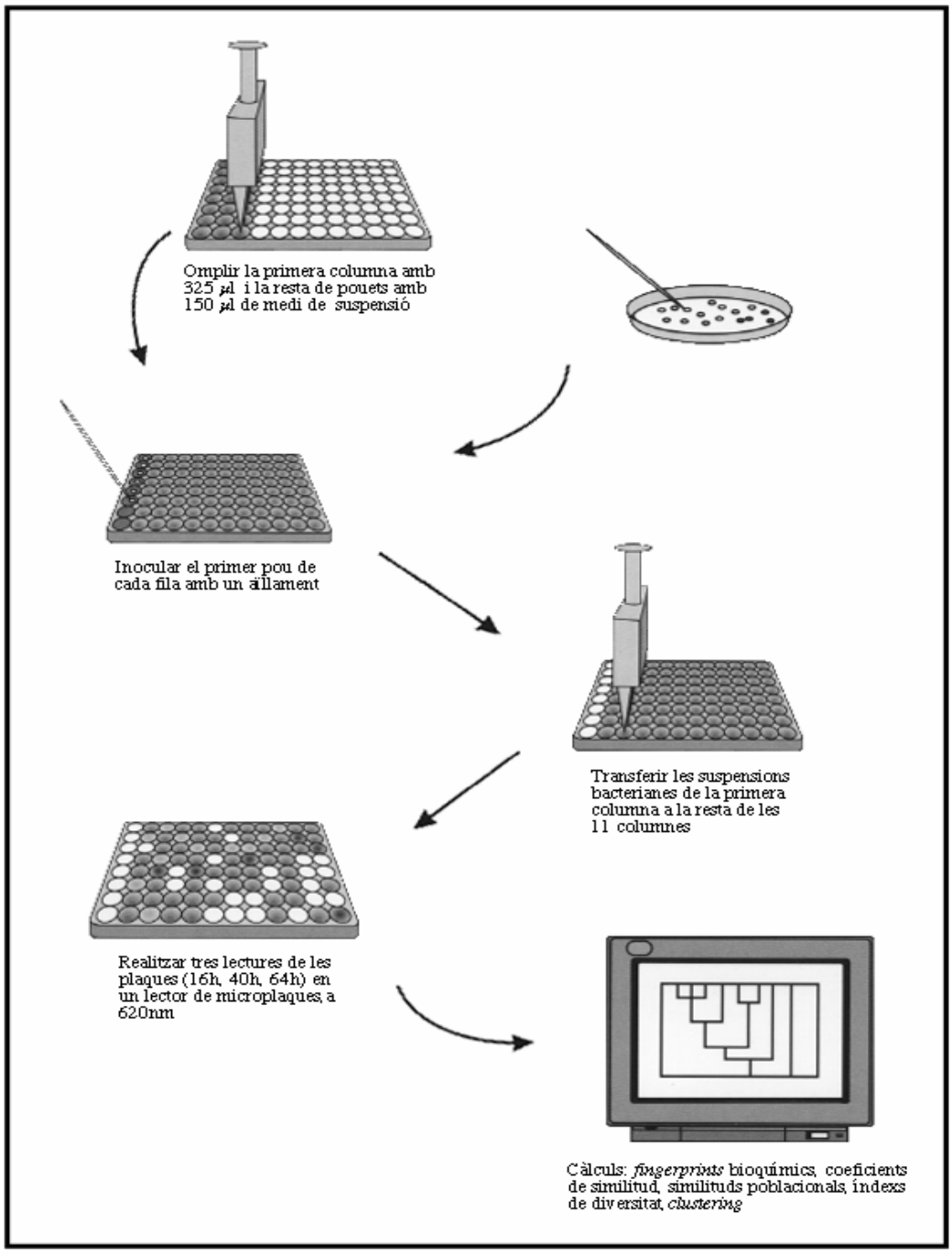
Pou número	PhP-RE	PhP-RF
1	Pou d'inoculació	Pou d'inoculació
2	Cel·lobiosa	L-Arabinosa
3	Lactosa	Lactosa
4	Ramnosa	Melbiosa
5	Desoxiribosa	Melezitosa
6	Sucrosa	Rafinosa
7	Sorbosa	Inositol
8	Tagatosa	Sorbitol
9	D-Arabitol	Manosa
10	Melibionat	Gal-Lacton
11	Gal-Lacton	Amigdalina
12	Ornitina*	Gluconat

* pou amb pH inicial àcid. La utilització d'aquest substrat produeix una alcalinització del medi. Gal-Lacton : Àcid D-(-) Galactònic-γ-lactona.

2.1 - Obtenció dels perfils bioquímics.

Les soques aïllades de coliforms fecals i enterococs a fenotipar s'incuben tota la nit en plaques d'un medi ric, en aquest cas Tryptic Soy Agar (TSA) (Difco, Detroit, U.S.A.) per coliforms i Brain Heart Infusion Agar (BHIA) (Difco) per enterococs, a 44ºC i 37ºC respectivament per tal de tenir cultius frescos. També seria possible fenotipar directament les colònies presents en les plaques dels medis selectius utilitzats pel seu aïllament, mFC agar (Difco) i m-*Enterococcus* Agar (MEA) (Difco) respectivament, però és important que les condicions siguin sempre les mateixes per a poder comparar els resultats amb els obtinguts en d'altres estudis o lots. En aquest estudi es va optar per la primera opció, ja que permet controlar que realment es treballa amb cultius purs. Mitjançant hisops estèrils s'inoculen les microplaques, resuspenent la càrrega densa, que es recull amb l'hisop d'un cultiu fresc, al primer pou de cada línia de la placa. Aquestes plaques s'han omplert prèviament i d'acord amb les instruccions dels fabricants, tal i com està descrit per **Kühn i Möllby** (**1993**), amb un brou de suspensió que conté 0,2% de peptona, 0,05% d'extracte de llevat, 0,5% de NaCl i 0,011% de blau de bromotimol en el cas del enterococs i 0,1% de peptona i 0,011% de blau de bromotimol pels coliforms fecals. A partir de la suspensió cel·lular feta en el primer pou de les microplaques es dispensen alíquotes de 25 µl a tots els pous de la mateixa fila (Figura 1). Les microplaques s'incuben a 37ºC pels dos grups bacterians. El creixement dels diferents pous es mesura utilitzant un espectrofotòmetre a 620 nm. Aquestes lectures es fan a les 7h, 24h i 48h pels coliforms fecals i a les 16h, 40h i 64h pels enterococs. Els perfils bioquímics es calculen d'acord amb **Kühn i col.** (**1991**) tal i com s'explica a continuació.

Figura 1 Procediment esquemàtic del tipatge bioquímic bacterià mitjançant el Phene-Plate System (PhP-Plate Microplates Techniques AB, Sweden).



Els valors d'absorbància de cadascuna de les 3 lectures són processats pel PhPWin software de la següent manera: de forma automàtica es multipliquen els valors de l'absorbància per 10, donant valors entre 0 i 30 per a cada reacció. Valors baixos indiquen reaccions acídiques (color groc), mentre que valors alts indiquen reaccions alcalines (color blau). El canvi de coloració es produeix quan l'indicador de pH contingut en la solució de suspensió vira per l'activitat metabòlica de la soca analitzada. Un cop feta la darrera lectura es calcula, per cada soca, el promig de les tres absorbàncies, donant lloc a 11 valors que oscil·len entre 0 i 30. Aquests 11 valors es corresponen a la capacitat de la soca analitzada per utilitzar els diferents substrats distribuïts en els pous, i defineixen el perfil bioquímic quantificat de cada una de les soques analitzades. Al fer 3 lectures a diferents temps, no només es valora la capacitat de metabolització dels diferents substrats, sinó també la cinètica d'aquesta metabolització.

Per cada mostra, els perfils bioquímics de totes les soques són comparats entre ells dos a dos, obtenint el coeficient de correlació (r) que indica la similitud entre cada parell de soques independentment de la resta de soques. Aquest valor oscil·la entre +1 i –1. Un valor proper a +1 indica que les dues soques comparades tenen un perfil bioquímic similar. A partir dels coeficients de correlació de totes les soques s'obté una matriu de similitud per aquell grup poblacional analitzat, a partir de la qual s'elabora el dendrograma més ajustat als coeficients de correlació calculats mitjançant el mètode UPGMA (*unweighted-pair groups method*) amb relacions de mitjanes (*average linkage*) seguits de l'agrupació de correlacions i coeficients Sp (**Kühn i col. 1991**; **Sneath i Sokal 1973**). El dendrograma comença agrupant les soques amb una Sp més elevada. Posteriorment es construeix una nova matriu de similitud en la que les soques agrupades en el primer pas es consideren com una sola. Aquest procediment es repeteix fins que totes les soques queden agrupades. Per defecte es considera que dues soques pertanyen a un mateix grup fenotípic o fenotip, és a dir, que comparteixen el mateix perfil bioquímic, quan presenten un coeficient de correlació superior a 0,975.

L'esquema de la Figura 2 representa els passos explicats fins aquí.

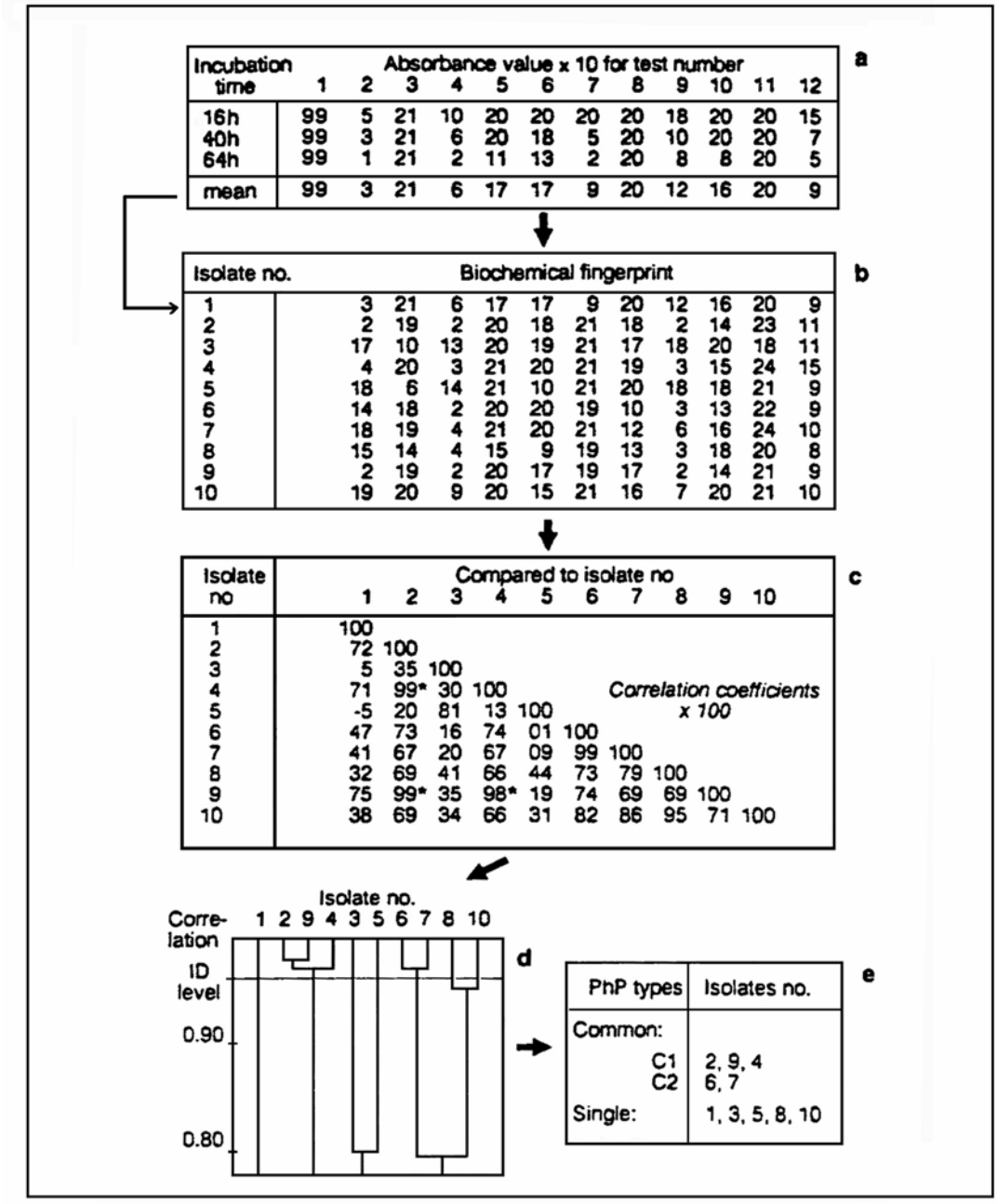


Figura 2 Procediment per a identificar grups fenotípics: a) càlcul del perfil bioquímic per a una soca davant dels 11 tests diferents; b) perfils bioquímics de 10 soques analitzades pels 11 test diferents; c) matriu de similitud obtinguda a partir de les comparacions, dos a dos, dels perfils bioquímics de b (les similituds estan expressades com a coeficients de correlació x 100. Els coeficients de correlació entre les soques del grup majoritari C1 s'indiquen amb un *); d) dendrograma obtingut a partir de la matriu de similitud; e) grups fenotípics identificats entre les 10 soques analitzades per a un determinat nivell d'identificació (ID *level* = 0,975).

2.2 - Anàlisis estadístiques.

2.2.1 - La diversitat poblacional.

La diversitat fenotípica de les poblacions bacterianes es mesura amb l'índex de diversitat (Di) de Simpson (**Atlas 1984; Hunter i Gaston, 1988**). Aquest índex oscil·la entre 0 (diversitat mínima) i 1 (diversitat màxima). Un valor de Di elevat indica que la població bacteriana està formada per molts grups fenotípics, mentre que si és baixa indica que determinats grups fenotípics dominen aquella població. Matemàticament, aquest índex correspon a la probabilitat que dues soques escollides a l'atzar dins d'una determinada població, no pertanyin a un mateix grup fenotípic. Es calcula mitjançant la fórmula:

$$1- \Sigma[N_i \times (N_i-1)] / [N \times (N-1)]$$

On N_i és el nombre de soques d'un mateix grup fenotípic " i " i N és el nombre total de soques.

Exemple:

La població X és formada per 6 soques: ●,●,●, □,□,□. Les figures iguals representen aquelles soques amb perfils bioquímics amb uns coeficients de correlació superiors a 0,975, és a dir, soques que estan dins del mateix grup fenotípic. La població Y està formada també per 6 soques: ●,●,●, □, ♫,♣.

Per calcular la probabilitat que a l'escollir a l'atzar 2 soques de la població X, aquestes no pertanyin al mateix grup fenotípic podem fer la següent aproximació:

Possibles combinacions: ●●, ●□, □●, □□. La probabilitat de la primera combinació seria 3/6 x 2/5; la de la segona 3/6 x 3/5; la de la tercera 3/6 x 3/5 i la quarta

3/6 x 2/5. Així la probabilitat que les 2 soques escollides a l'atzar siguin iguals seria la suma de la primera probabilitat i la quarta (0,2+0,2 = 0,4).

En aquest primer cas, si apliquem la fórmula de Simpson obtenim:

1-[3 x (3-1)+3 x (3-1)/ 6 x (6-1)] = 0,6

En el segon cas, on hi ha una major diversitat, tindríem que la probabilitat que dues soques escollides a l'atzar en la població Y pertanyin al mateix fenotip (combinació ●●) seria de 3/6 x 2/5 = 0,2. Aplicant la fórmula de Simpson s'obté:

1- [3 x (3-1)+1 x (1-1)+1 x (1-1)+1 x (1-1) / 6 x (6-1)] = 0,8

i per tant els valors de Di són superiors als del primer cas considerat (població X).

2.2.2 - La similitud poblacional.

La similitud fenotípica entre poblacions es calcula mitjançant el coeficient de similitud poblacional (Sp). Aquest coeficient és una mesura de la proporció de soques amb el mateix perfil bioquímic presents en dues poblacions bacterianes donades (Kühn, 1985). És elevat (valor màxim igual a 1) si les poblacions comparades estan formades pels mateixos grups fenotípics i aquests es troben en proporcions idèntiques en les dues poblacions. És baix (valor mínim igual a 0) si les soques d'una i altra població no comparteixen cap perfil bioquímic. Es calcula mitjançant la següent fórmula.

$Sp = (S_x+S_y)/2$

On S_x i S_y representen la similitud de la població X en la població Y, i a la inversa. Es calculen segons aquestes fórmules:

$$S_x = \Sigma\, qx_i/N_x \qquad\qquad S_y = \Sigma\, qy_i/N_y$$

$$qx_i = Px_i/Py_i \qquad qy_i = Py_i/Px_i$$

N_x = nombre de soques de la mostra x

N_y = nombre de soques de la mostra y

i = nombre de la soca (identificatiu, ordinal) dins de la població x. Va de 1 fins N_x.

Px_i (de la població X) = proporció de les soques de la població X pertanyents al mateix grup fenotípic que la soca x_i respecte al total de les soques de la població X.

Py_i (de la població X) = proporció de les soques de la població Y pertanyents al mateix grup fenotípic que la soca x_i respecte al total de les soques de la població Y.

Px_i (de la població Y) = proporció de les soques de la població X pertanyents al mateix grup fenotípic que la soca y_i respecte al total de les soques de la població X.

Py_i (de la població Y) = proporció de les soques de la població Y pertanyents al mateix grup fenotípic que la soca y_i respecte al total de les soques de la població Y.

q_{xi} ha de ser més petit o igual a 1; en cas contrari q_{xi} serà igual a $1/q_{xi}$
q_{yi} ha de ser més petit o igual a 1; en cas contrari q_{yi} serà igual a $1/q_{yi}$

Exemple:

L'índex de similitud Sp entre la població X de l'exemple anterior (●,●,●, □,□,□) i la població Y (●,●,●, □, ♫,♣) seria el següent:

Població X (els nombres indiquen l'ordinal i)	**Població Y** (els nombres indiquen l'ordinal i)
●1 ●2 ●3 □4 □5 □6	●1 ●2 ●3 □4 ♫5 ♣6

Px_i	3/6	3/6	3/6	3/6	3/6	3/6	Py_i	3/6	3/6	3/6	1/6	1/6	1/6
Py_i	3/6	3/6	3/6	1/6	1/6	1/6	Px_i	3/6	3/6	3/6	3/6	0	0
qx_i	1	1	1	1/3	1/3	1/3	qy_i	1	1	1	1/3	0	0

$S_x = [1+1+1+1/3+1/3+1/3] / 6 = 12/18$

$S_y = [1+1+1+1/3+0+0] / 6 = 10/18$

$Sp = (S_x + S_y) /2$

$Sp = [(12/18) + (10/18)] / 2 = 0,61$

Cal fer notar que a l'hora d'interpretar els resultats de Sp és important tenir en compte la diversitat i el nombre de soques de les poblacions analitzades. Quan l'índex Di és superior a 0,9 i el nombre de soques fenotipades per població és d'entre 20 i 30, la Sp difícilment superarà valors de 0,5 ja que en rèpliques d'una mateixa mostra d'aquestes característiques s'obtenen valors de Sp propers a 0,5.

A partir de la matriu dels coeficients Sp obtinguda al comparar les mostres o els grups poblacionals establerts arbitràriament, es poden representar gràficament les relacions entre les seves poblacions bacterianes. Seguint el mètode UPGMA descrit a l'apartat 2.1 es poden dibuixar dendrogrames com els descrits a la Figura 2(d). En aquests cas, a la Figura 3, cada mostra està representada a l'eix vertical i les similituds entre poblacions (coeficients Sp) a l'eix horitzontal. Les diferents poblacions estan connectades per línies verticals al nivell de similitud que mostren entre elles, de manera que com més a la dreta es trobin aquestes, més similars són les poblacions.

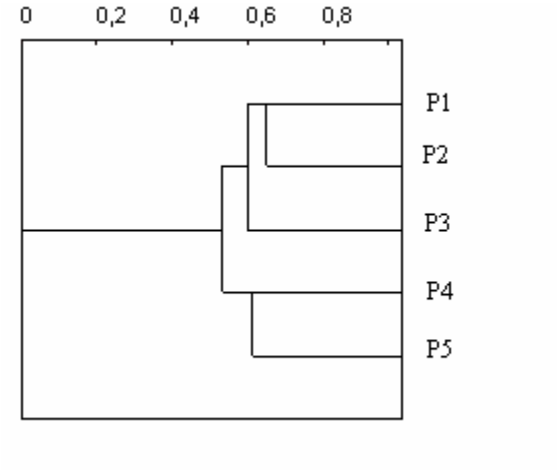


Figura 3 Dendrograma on es representa gràficament la similitud mitjançant el mètode UPGMA de 5 poblacions: P1, P2, P3, P4 i P5.

2.3 – Presa de mostres, pretractament i determinació de la càrrega microbiana de les aigües analitzades.

Per tal de comparar les poblacions de coliforms fecals i d'enterococs de les aigües residuals mitjançant el Phene Plate-System, es van prendre mostres de cinc estacions depuradores d'aigües residuals (en endavant EDARs) abans i després del seu tractament: les EDARs de Tona (1), Taradell (2), Ripoll (3), Gavà (4) i Sant Adrià del Besòs (5). També es van prendre mostres del riu Ter on s'abocava les aigües tractades de l'EDAR de Ripoll, 50 metres aigües amunt de l'emissari, 50 metres riu avall i 2 Km riu avall així com de fangs en dos estadis diferents de l'EDAR de Taradell. Segons les dades facilitades pels gestors d'aquesta planta, el residu sòlid a 104ºC d'aquestes mostres era d'entre 0,1 i 0,3 g/100 ml en el cas de les aigües residuals, 0,4 i 0,6 g/100ml en els fangs de recirculardització i entre 14 i 15 g/100ml en el cas dels fangs premsats. Les aigües tractades de la depuradora de Ripoll que s'aboquen al riu Ter representen al voltant d'un 15,8% del cabal mínim anual del riu, un 0,05% del màxim i un 0,9% del cabal mitjà anual, degut al règim mediterrani d'aquest riu. L'enumeració de les EDARs correspon a les dades de la Taula 1 on es detallen també les característiques de cada planta. Es va determinar la càrrega d'aquestes poblacions microbianes i posteriorment es va estudiar la seva composició i estructura. A més a més es va determinar la càrrega d'espores de clostridis sulfit-reductors i es va valorar la presència de les poblacions d'enterococs resistents a la vancomicina i a l'eritromicina.

EDAR	Cabal (m^3dia^{-1})	Població	Tractament biològic	Temps de retenció	Origen de l'aigua residual
1	2.160	6.000	Fangs activats	1,5 dies	Mixt humà i animal
2	2.000	5.000	Fangs activats	2,2 dies	Predominantment humà
3	7.500	16.000	Fangs activats	1,04 dies	Predominantment humà
4	72.000	390.000	Fangs activats	4 hores	Predominantment humà
5	670.000	1.500.000	No	1 hora	Predominantment humà

Taula 2. Principals característiques de les 5 EDARs: cabal en m^3dia^{-1}, població a la que dóna servei, utilització de tractament biològic (fangs activats) i temps de retenció hídrica.

Les mostres es van prendre durant un període de 2 anys assegurant-ne la representativitat de cada punt segons normatives (**Anon. 1980**) en hores de màxima aportació i en les diferents estacions de l'any, utilitzant recipients estèrils (**Anon. 1991**). Seguint els protocols estandarditzats es van transportar a 4ºC i es van analitzar abans de 6 hores (**Anon. 1994**).

A partir de les mostres es van realitzar bancs de dilucions decimals en PBS (solució salina de tampó fosfat) a pH 7,0 per cada una de les poblacions microbianes a quantificar. Les mostres de fangs premsats es van homogeneïtzar en PBS a pH 7,0 (solució 1:10 pes/volum) mitjançant agitació durant 30 minuts, mentre que les de fangs de recirculardització es van homogeneïtzar sense dilució i amb la mateixa agitació. Després de 5 minuts en repòs, es va analitzar el sobrenedant de la mateixa manera que les mostres d'aigua.

Les rèpliques de les diferents dilucions decimals van ser posteriorment filtrades a través de membranes de 0,45 μm de porus (Millipore, Bedford, U.S.A.). Una part d'aquestes membranes es disposaren sobre plaques de m-FC Agar (Difco, Detroit, U.S.A.) i es van incubar a 44ºC i durant 24h, en el cas dels coliforms fecals (**Anon. 1997a**). El nombre de colònies blaves va permetre determinar la càrrega de coliforms fecals de cada mostra (**Grabow 1990**). Pel que fa als enterococs, les membranes es van incubar primer 2 hores a 37ºC en el medi ric Brain Heart Infusion Agar (BHIA) (Difco) per a intentar recuperar els bacteris estressats (**Anon. 1998**). A continuació i seguint el protocol I.S.O. per a la detecció i l'enumeració d'enterococs (**Anon. 1997b**), es transferiren aquestes membranes respectivament sobre m-*Enterococcus* Agar (MEA) (Difco), MEA suplementat amb 8 mg l^{-1} d'eritromicina i MEA suplementat amb 8 mg l^{-1} de vancomicina, per tal d'enumerar els enterococs totals, els enterococs resistents a la vancomicina i els enterococs resistents a l'eritromicina respectivament (en l'apartat 3 d'aquesta introducció s'explica perquè es va escollir l'enumeració d'aquestes resistències). Totes aquestes plaques es van incubar a 37ºC durant 48h. A més a més, es va realitzar un enriquiment de la mostra inoculant 10 ml de cadascuna en 10 ml de brou Enterococcosel™ (Becton Dickinson, Cockeysville, U.S.A.) a doble concentració suplementat amb 16 mg l^{-1} de vancomicina, incubant-ho a 37ºC durant 24h. Alíquotes de 10 μl dels enriquiments amb creixement es varen sembrar en plaques de MEA

suplementades amb 8 mg l^{-1} de vancomicina. Per tal de determinar la capacitat d'hidrolitzar l'esculina, les membranes es van transferir sobre Bile Esculin Agar (BEA) (Difco) i les colònies crescudes directament sobre la placa de MEA suplementada amb 8 mg l^{-1} de vancomicina (procedents del brou d'enriquiment) es van ressembrar també en BEA. Les plaques s'incubaren durant 2h a 44ºC i posteriorment es comptaven com a enterococs aquelles colònies amb coloració fosca que ens indicava que havien hidrolitzat l'esculina (**Figueras i col. 1998**; **Manero i Blanch 1999**).

La concentració d'espores de clostridis sulfit-reductors es va determinar sotmetent les mostres (el sobrenedant de l'homogenat en el cas dels fangs) a un xoc tèrmic a 80ºC durant 10 minuts (**Handford 1974**). Posteriorment, es realitzaren dilucions decimals en PBS a pH 7,0 i 1 ml de cadascuna d'elles es va inocular en 50 ml de Sulfit-Polymyxin-Sulfadiazin-A Agar (SPS) (Scharlau, Barcelona, Espanya) en estat líquid, deixant-lo solidificar en tubs de 50 ml. Aquests tubs es van incubar a 44ºC durant 24 h. Es van comptar les colònies que manifestaven la seva capacitat de reduir el sulfits adquirint una coloració negra.

2.4 - Determinació dels perfils bioquímics de les soques de coliforms fecals i de les d'enterococs aïllades.

Es van aïllar un màxim de 24 soques de coliforms fecals i 24 d'enterococs a partir de les membranes de cada mostra que presentaven un nombre de colònies entre 30 i 100. Aquest nombre de soques és representatiu de les poblacions bacterianes d'una mostra enumerada en un medi sòlid (**Bianchi i Bianchi 1982**; **Kühn i col. 1997**). A més a més, es van aïllar fins a un màxim de 10 soques per mostra provinents de les membranes incubades a 8E i 8V i del brou d'enriquiment a 8V. Cadascuna de les soques de coliforms fecals i enterococs aïllades es va sembrar en plaques de BHIA, incubant-se durant la nit a 44ºC i 37ºC respectivament. Mitjançant escuradents estèrils es va agafar un inòcul dens de cultiu fresc de coliforms fecals i enterococs per tal d'inocular respectivament els pouets de la primera columna de les plaques PhP-RE i PhP-RF (PhP-Plate Microplates Techniques AB, Sweden). Tal i com ja s'ha explicat anteriorment en l'apartat 2.1.

2.5 - Índex de diversitat i similitud poblacional del coliforms fecals i els enterococs aïllats.

Es varen calcular els índex de diversitat i de similitud poblacional, tal i com s'ha explicat en l'apartat 2.2, per a cadascun dels llocs de mostreig dels diferents estudis realitzats i per a les poblacions de coliforms fecals i d'enterococs. També es varen calcular per a les poblacions d'enterococs resistents a l'eritromicina i a la vancomicina.

2.6 - Agrupació fenotípica de les soques aïllades i identificació de les soques representants dels principals grups fenotípics.

Els resultats obtinguts en les lectures de les microplaques PhP-RF i PhP-RE varen permetre determinar per a cada soca un perfil de característiques bioquímiques que anomenem perfil bioquímic. Les diferents soques aïllades es van agrupar, en base als seus perfils bioquímics, constituint els grups fenotípics. Cada grup està constituït per totes aquelles soques que presenten un coeficient de correlació dels seus perfils bioquímics superior al 0,975. Tots aquells grups de coliforms fecals que representaven més del 5% d'una mostra donada, o més d'un 10% en el cas del enterococs, es van considerar en els estudis posteriors. El motiu del criteri de selecció entre coliforms fecals i enterococs respon als índexs de diversitat de cadascuna d'aquestes poblacions. Dins de cada grup fenotípic, aquella soca que presentava el valor de mínima similitud respecte a les altres soques no pertanyents al grup i la mitjana de similaritat més elevada respecte les soques del propi grup es va considerar com a soca representativa d'aquell grup fenotípic. Les soques representats es van congelar en brou Brain Heart Infusion (BHI) (Difco) suplementat amb un 10% de glicerol a una temperatura de –70ºC per a la seva posterior identificació. Les soques representants dels grups fenotípics de coliforms fecals amb més de 20 soques es van identificar mitjançant les galeries API 20E (BioMérieux, la Balme, France). Els representats dels grups fenotípics d'enterococs (és a dir, tots aquells perfils bioquímics que representaven més del 10% d'una determinada mostra i que havien estat congelats) es van identificar utilitzant les claus d'identificació descrites per **Manero i Blanch** (**1999**). Aquesta identificació va servir per anomenar els diferents grups fenotípics. Els enterococs presents en baixes proporcions pertanyents a les espècies filogenèticament molt properes com *Ent. hirae* i *Ent. durans* o *Ent. gallinarum*, *Ent. casseliflavus* i *Ent. flavescens* (**Behr i col. 2000**) es van considerar com a un únic grup. Tots aquelles soques amb un perfil bioquímic minoritari (en el cas dels coliforms fecals totes aquelles amb un perfil compartit per menys de 20 soques entre les més de 2.000 caracteritzades bioquímicament al llarg dels estudis) es van classificar en el grup d'"altres". El percentatge de soques incloses en grups fenotípics pertanyents a la mateixa espècie o agrupació d'espècies es va calcular per a cadascun dels punts analitzats.

3–Els enterococs i la resistència als glicopèptids i als macròlids.

A més a més del paper com a indicadors que tenen els enterococs, la teràpia antimicrobiana moderna ha classificat algunes espècies d'enterococs entre els patògens nosocomials més importants (**Robredo i col. 1999**). En els darrers 20 anys s'ha detectat un increment de la resistència a antibiòtics per part dels enterococs. La capacitat per a desenvolupar resistència a diferents antibiòtics, i especialment quan es tracta dels més utilitzats a nivell clínic contra els enterococs, planteja importants problemes. Com a exemple s'ha descrit que *Enterococcus faecium* resistents a la vancomicina (VREF) són també resistents a múltiples antibiòtics, la qual cosa dificulta el seu tractament en infeccions nosocomials (**Willems i col. 2000**).

La vancomicina és un antibiòtic de la família dels glicopèptids que són produïts per diferents espècies d'*Actinomyces*, tot i que actualment es pot sintetitzar químicament. Aquests antibiòtics actuen sobre l'enllaç D-alanina-D-alanina terminal de la subunitat de la N-acetil-muramil-pèptid de la paret dels bacteris gram positiu en creixement. Hi ha múltiples gens que proporcionen resistència a aquest antibiòtic. Existeixen diferents exemples dels mecanismes de resistència a la vancomicina, com ara la modificació dels enllaços sobre els que actua l'antibiòtic. S'han definit almenys 5 fenotips de resistència a aquest glicopèptid en els enterococs anomenats respectivament **VanA**, **VanB**, **VanC**, **VanD** i **VanE**. Aquesta classificació es basa en la concentració a la que presenten resistència cadascun d'ells (**Méndez-Álvarez i col. 2000**). Els dos primers són els més resistents a altes concentracions de vancomicina. Els gens que proporcionen aquest fenotip es troben en els transposons Tn*1546* i Tn*1547* que poden ser en plasmidis o també presentar-se inserits en el cromosoma bacterià. El fenotip **VanC** correspon a una baixa resistència a la vancomicina, però és intrínseca en espècies com *E. gallinarum*, *E. casseliflavus* i *E. flavescens*. El fenotip **VanD** s'ha trobat en 4 soques d'*E. faecium*. S'expressa constitutivament a la soca *E. faecium* BM4339, mentre que en les altres soques és induït per la vancomicina. Els gens que en determinen aquest fenotip es localitzen al cromosoma i no són transferibles a d'altres enterococs (**Ostrowsky i col. 1999**; **Périchon i col. 1997**). El fenotip **VanE** s'ha descrit en la soca de *E. faecalis* BM44005. Aquests dos darrers fenotips corresponen a un grau de

resistència intermèdia entre els dos primers (**VanA** i **VanB**) i **Van C** (**Méndez-Álvarez i col. 2000**).

Una de les teràpies alternatives a la vancomicina la constitueixen els macròlids-lincosamida-estreptogramina (MLS). Els macròlids de caràcter bàsic es caracteritzen químicament per la possessió d'un gran anell lactònic al que s'uneixen ponts glucosídics aminosucres. S'obtenen del gènere *Streptomyces*, excepte la rosaramicina que és produïda per *Micromonospora*. Actuen inhibint la síntesi proteica, ja que s'uneixen a la subunitat ribosòmica 50S impedint la translocació i al mateix temps l'elongació de la cadena peptídica. Les lincosamides també s'uneixen a la subunitat 50S dels ribosomes impedint la transpeptidació (transferència de l'aminoàcid de l'ARNm a la cadena peptídica). Les estreptogramines inhibeixen la formació de l'enllaç peptídic. En bacteris gram positius hi ha tres mecanismes diferents d'adquisició de resistència al MLS: la modificació de la diana, la inactivació del fàrmac o l'expulsió activa del mateix. En el primer cas, una simple alteració del 23S RNAr confereix resistència creuada als tres antibiòtics, mentre que la inactivació del fàrmac només confereix resistència a cadascun d'ells. Pel que fa als mecanismes de bombeig, els gens ***mefA***, ***mefE***, ***msrA*** i ***mreA*** estan implicats en l'expulsió dels antibiòtics en els bacteris gram positius. Concretament, els gens ***mef*** i ***mreA*** estan associats amb la resistència al macròlid mentre que ***msrA*** ho està, a més, a la resistència de l'estreptogramina B (**Portillo i col. 2000**).

En el cas dels enterococs s'han descrit resistències conferides pels gens ***erm***, que codifiquen per una metilasa, pels ***mef***, que codificarien per presumptes bombes d'eritromicina (**Luna i col. 1999**) i pels ***msr*** que codificarien per una aminoglicòsid acetil-transferasa (**Portillo i col. 2000**). Precisament l'eritromicina ha estat un dels macròlids més utilitzats com a teràpia alternativa a la vancomicina. Un anàleg d'aquesta, la tilosina, s'ha utilitzat també com a promotor del creixement en animals, i ha estat assenyalada com el possible origen de la resistència a l'eritomicina en enterococs (**Jackson i col. 2004)**. Paral·lelament, un altre promotor del creixement en sistemes intensius de producció animal, l'avoparcina, anàleg a la vancomicina, ha estat assenyalat com el possible origen de la resistència a aquest darrer antibiòtic (**Stobbering i col. 1999; Aarestrup 1995; Klare i col. 1995**).

Els enterococs resistents a la vancomicina (VRE) es van detectar per primera vegada a França, el 1986 (**Leclercq i col. 1988**; **Uttley i col. 1988**) i han esdevingut una important causa d'infeccions nosocomials a tot el món. El 1993 s'aïllen enterococs resistents a la vancomicina del tipus *VanA*, en femtes d'animals de granja (**Bates i col. 1993**). També un estudi alemany documenta la detecció d'*Enterococcus faecium* resistent a la vancomicina (*VanA*) en aigües residuals de depuradora (**Klare i col. 1993**). El 1995 uns estudis realitzats per grups danesos i alemanys descriuen la colonització en porcs i cavalls de VRE, així com també en les comunitats humanes properes, suggerint així la possibilitat que l'ús de l'avoparcina com a promotor del creixement produís una selecció positiva dels VRE (**Aarestrup 1995; Klare i col. 1995**). La transmissió dels VRE d'origen animal cap als humans a través de la cadena alimentària va ser proposada com la via més plausible. El contacte entre animals exposats a l'avoparcina i els humans en explotacions ramaderes també es va apuntar en diversos estudis (**Kruse i Rovik 1996**; **Van den Bogaard i col. 1997**). En estudis posteriors es constata la presència de VRE en granges 3 anys després de la prohibició de l'avoparcina (**Borgen i col. 2000**). Però mentre que els estudis realitzats a Europa apunten a la utilització de l'avoparcina com a promotor del creixement en explotacions ramaderes, les línies d'investigació als Estats Units apunten a la utilització massiva d'antibiòtics a nivell clínic com a principal causa de la proliferació dels VRE (**Goossen 1998**). Així per exemple estudis realitzats a Estats Units per **Boyle i col.** (**1993**) mostren que els aïllaments d'enterococs fets en pacients en un hospital al llarg de 3 anys presenten un increment de la resistència a la vancomicina del 14% el 1990, i del 75% el 1992.

L'aïllament de soques resistents a aquests antibiòtics tant en entorns hospitalaris com en produccions ramaderes intensives, i l'ús de molècules anàlogues com a promotors del creixement animal que es consideren implicades en l'aparició de les resistències, ens va fer plantejar la valoració de la presència de les soques resistents en aigües residuals. Aquestes soques a més a més, ens resultaven un bon marcador com a eina per a valorar i complementar els resultats del biotipat bioquímic amb el Phene-Plate System en l'estudi de l'eliminació selectiva de poblacions bacterianes i els canvis de la diversitat en entorns aquàtics.

OBJECTIUS GENERALS.

El treball realitzat en aquesta tesi es centra en l'anàlisi de l'estructura i la composició de les poblacions de coliforms fecals i enterococs en les aigües residuals de diferents orígens, mitjançant l'estudi de la seva diversitat. S'avaluen les variacions d'aquests paràmetres després dels diferents processos de depuració, així com en els fangs d'una planta de tractament d'aigües residuals i en el riu receptor de les aigües tractades en una de les EDARs. Atenent a la importància que té l'aparició de soques resistents a diferents antibiòtics en malalties de tipus nosocomial i la potencial transferència d'aquesta resistència per la cadena tròfica, i particularment a través de les aigües residuals dintre del cicle de l'aigua, s'ha estudiat la persistència dels enterococs resistents a la vancomicina (VRE) i a l'eritromicina (ERE) després del tractament d'aigües residuals. Aquestes subpoblacions a més, serveixen com a marcadors en l'estudi de l'estructura de les poblacions.

Els principals objectius d'aquesta tesi són:

1. Anàlisi de la diversitat i dels clons poblacionals de coliforms fecals i enterococs d'aigües residuals entre diferents plantes de tractament

2. Avaluació de la presència i diversitat de les poblacions d'enterococs resistents a la vancomincia i a l'eritromicina en les aigües residuals de diferents depuradores.

3. Determinació i comparació de la composició i l'estructura de les poblacions de coliforms fecals i enterococs entre el riu receptor d'un efluent d'una depuradora, amb les aigües residuals tractades d'aquesta.

4. Valoració de la capacitat d'autodepuració del riu en el tram estudiat, atenent a les poblacions d'aquests dos indicadors bacterians.

5. Avaluació de la persistència i de les modificacions en l'estructura de les poblacions de coliforms fecals i enterococs en els fangs d'una depuradora.

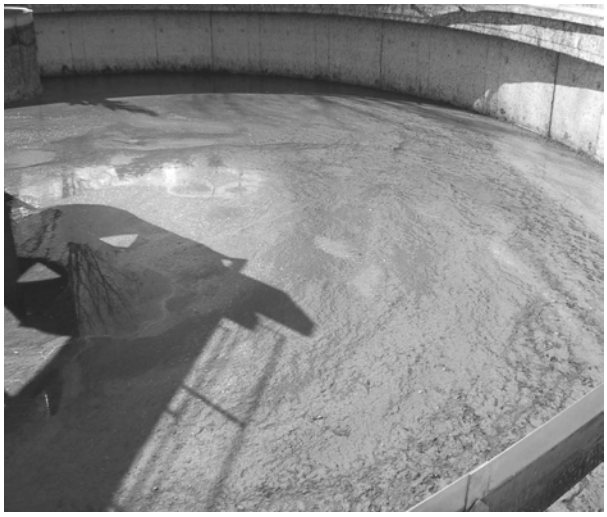
6. Anàlisi de la presència de poblacions d'enterococs resistents a la vancomicina i l'eritromicina en diferents fangs d'una depuradora.

7. Comparació de les poblacions d'enterococs en les aigües residuals municipals i d'hospitals en diferents regions europees.

Els objectius 1 i 2 s'aborden en el capítol 1; els objectius 3 i 4 en el capítol 2; el 5 i 6 en el capítol 3 i el 7 en el capítol 4.

Els resultats obtinguts durant la realització d'aquesta tesi s'exposen en 4 capítols diferenciats. Els capítols I, II i IV consten d'un article publicat. En el tercer capítol es presenten les dades que apareixeran en un article sotmès per a la seva publicació.

RESULTATS.



Capítol 1

The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants

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RESUM DEL CAPÍTOL

Els coliforms fecals i els enterococs han estat emprats extensament com a indicadors de contaminació fecal. També s'ha estudiat la seva persistència en l'ambient i davant de diferents factors físics, químics o biològics. Els resultats descrits en la bibliografia són coincidents quan s'avalua l'efecte d'un sol factor. No obstant, quan s'estudien situacions complexes en les que intervenen molts factors, els resultats obtinguts no són tant homogenis i la seva explicació sovint no és senzilla. Fins al moment, no es disposa de gaires treballs sobre la diversitat dintre de les poblacions d'aquests indicadors en aigües residuals, i menys al llarg d'un procés de tractament. D'altre banda l'aparició d'enterococs resistents a la vancomicina (VRE) i a l'eritromicina (ERE) ha esdevingut un factor preocupant en els darrers anys. L'objectiu d'aquest capítol fou el d'analitzar els canvis en l'estructura i la composició de les poblacions de coliforms fecals, enterococs, VRE i ERE en les aigües residuals de cinc plantes de tractament en funció de la procedència de les aigües residuals i dels diferents processos de depuració. També es va determinar la càrrega d'espores de clostridis sulfit-reductors com un element més de referència degut a la seva elevada persistència en l'ambient.

La càrrega microbiana de les aigües residuals crues analitzades presentava unes proporcions constants dels 3 indicadors estudiats així com de VRE i ERE. Per contra, en l'aigua tractada hi havia un canvi d'aquestes proporcions pel que fa a les espores de clostridis sulfit-reductors. Aquesta variació era més acusada en aquelles depuradores amb un temps de retenció hídrica major. No obstant, l'índex de diversitat i el de similitud poblacional detectats amb la tècnica del Phene-Plate system foren elevats, tant entre poblacions d'aigües residuals dels diferents punts com entre poblacions de l'aigua

residual crua i la tractada. Tot i la elevada similitud, les poblacions de la depuradora amb més aportació fecal d'origen animal fou la menys semblant a la resta.

APORTACIÓ PERSONAL AL TREBALL

L'autor d'aquesta tesi ha realitzat el disseny del mostreig, la presa de mostres, les anàlisis microbiològiques i l'anàlisi del resultats obtinguts amb la col·laboració del Dr. Albert Manero Camps en la identificació de les soques d'enterococs i de la Dra. Marta Cerdà Cuéllar en el Phene-System, així com la supervisió del professor Anicet R. Blanch Gisbert.

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The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants

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ABSTRACT

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Aims: The changes in structure and composition of faecal coliforms and enterococcal populations in sewage from different treatment plants, and the elimination of vancomycin- and erythromycin-resistant enterococci (VRE and ERE, respectively) in these treatment plants was analysed to determine any selective reduction.

Methods and Results: Faecal coliforms, enterococci, VRE, ERE and spores of sulphite-reducing bacteria were enumerated using standard methods. Samples were enriched where necessary in order to isolate antibiotic resistant strains. The structure and composition of these bacterial populations were determined by biochemical fingerprinting and clustering analysis. High diversity and similarity indexes were detected among all the bacterial populations in raw and treated sewage, independently of their origin and the treatment processes employed. Antibiotic resistant strains were detected in all sewage tested and no selective reduction was observed.

Conclusions: The faecal coliforms and enterococci populations did not differ in the sewage samples studied. The vancomycin and erythromycin resistances of the enterococcal populations were similar in the sewage samples. Resistance to both antibiotics persisted after the treatment process independently of raw sewage flow, faecal origin or size of the human population contributing to sewage. However, sewage of mixed origin (human and animal) presented a lower similarity index for the two bacterial populations compared with that of the other human sewage analysed.

Significance and Impact of the Study: Although a significant reduction in bacterial populations was observed, the persistence of VRE and ERE strains in the same proportions in sewage suggests that there is no selective elimination of bacterial populations during the treatment processes. The ability of antibiotic resistance strains to survive sewage treatment systems should be considered in certain water reuse programmes.

Keywords: *Enterococcus*, erythromycin, faecal coliform, sewage, vancomycin.

INTRODUCTION

Faecal coliform, enterococci and spores of sulphide-reducing bacteria (SRB) have been widely used as indicators of faecal pollution (Anon. 1998). However, the persistence of these bacterial groups in the environment varies, so that the persistence of SRB spores is greater than that of enterococci, which in turn is greater than that of faecal coliforms in freshwater and marine water (Barcina *et al.* 1990, 1997; Sinton *et al.* 1994; Durán *et al.* 2002). Such rates of persistence are similarly observed when the groups are affected by a range of factors including: sunlight (Fujioka *et al.* 1981), temperature (Mocé-Llivina *et al.* 2003), ciliate predation (González *et al.* 1990) and chlorination (Tree *et al.* 2003). Likewise, these rates have also been reported in wastewater treatments (Chauret *et al.* 1999). However, a similar reduction in enterococci and faecal coliforms populations has been detected in secondary treatment systems,

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in a primary anaerobic lagoon, a physico-chemical treatment plant and wetland sediments (Hill and Sobsey 1998; Payment *et al.* 2001; Stenstrom and Carlander 2001; Vilanova *et al.* 2002).

By contrast, the different origin of pollutants in waters might result in a low degree of bacterial diversity with certain groups becoming predominant (Kühn *et al.* 1997; Gauthier and Archibald 2001). Yet, other studies have reported similar distributions of faecal coliform species independently of the sources of pollution (Brown and Tracey 1975; Mersch-Sundermann and Wundt 1987; Hill and Sobsey 1998). Furthermore, earlier studies examining the fate of faecal coliforms and enterococci populations in a rural treatment plant showed that the raw and treated sewage effluent, and the reception river waters had a similar composition (Vilanova *et al.* 2002). However, a similar origin of faecal pollution up-stream in the river could not be ruled out. This is supported by other studies in which enterococcal populations in the sewage from different countries were found to be largely similar (Blanch *et al.* 2003). This finding agrees with the distribution of the predominant species of *Enterococcus* reported by several authors world-wide (Sinton and Donnison 1994; Laukova and Juris 1997; Svec and Sedlacek 1999; Manero *et al.* 2002).

The effects of sewage treatment on the antibiotic resistance of coliform bacteria and enterococci have also been analysed. It has been suggested that the resistance patterns are transient characteristics, and that resistance may fluctuate as the sewage moves through the treatment plant (Andersen 1993; Mezrioui and Baleux 1994). Thus, the presence of antibiotic-resistant bacteria in natural waterways may contribute to the widespread dissemination of antibiotic resistance (Grabow and Prozesky 1976; Bell *et al.* 1981; Mach and Grimes 1982). VRE have been identified as a significant cause of hospital-acquired infection (Goossens 1998) and have been linked with animal production (Robredo *et al.* 1999; Stobbering *et al.* 1999; Teuber and Perreten 2000). Moreover, enterococci isolates of animal and human origin have been reported to carry erythromycin-resistant genes (Jensen *et al.* 1999; Aarestrup *et al.* 2002). Resistance to both antibiotics has been linked to mobile genes on conjugative transposons and plasmids (Luna *et al.* 1999; Méndez-Álvarez *et al.* 2000; Portillo *et al.* 2000). Consequently, resistance to these two antibiotics could potentially be transferred between bacterial populations in wastewater, particularly in sewage treatment plants which have a high concentration of bacteria of distinct faecal origin (Mezrioui and Baleux 1994).

This study analyses the changes in the structure and composition of faecal coliforms and enterococcal populations induced at different sewage treatment plants. In order to determine whether there was any selective reduction or increase in bacterial populations with treatment process, vancomycin- and erythromycin-resistant enterococci (VRE and ERE, respectively) were evaluated in raw and treated sewage. Additionally, the die-off rates of spores of SRB, faecal coliforms and enterococci were calculated. Faecal coliforms and enterococci strains were isolated and biochemical fingerprinting was performed to determine changes in these groups at different treatment plants and to compare sewages of different origin. Representative strains of the most abundant phenotypes were identified and the distribution of the main species was also evaluated for both bacterial groups.

MATERIALS AND METHODS

Sampling, pretreatment and enumeration of bacterial populations

Raw and treated sewage waters from five wastewater treatment plants were sampled several times over a 2-year period. These plants differed in their sewage flow, retention time, origin of sewage, availability of biological treatment and the number of inhabitants generating sewage (Table 1). Samples were collected and stored at 4°C in accordance with standard protocols (Anon. 1994, 1998). The enumeration of faecal coliforms and enterococci was performed by membrane filtration (Anon. 1997a,b) on 0·45 μm pore size

Table 1 Main characteristics of the five sewage treatment plants: flow in m^3 day^{-1}, conurbation served (population), presence of biological treatments, hydraulic retention time of wastewater and origin of the sewage

Plant	Flow (m^3 d^{-1})	Population	Treatment	Hydraulic retention time	Origin of waste
1	2160	6000	Activated sludge	1·5 days	Mixed human and animal
2	2000	5000	Activated sludge and simultaneous chemical precipitation (ferric chloride)	2·2 days	Mostly human
3	7500	16 000	Activated sludge and simultaneous chemical precipitation (ferric chloride)	1·04 days	Mostly human
4	72 000	390 000	Activated sludge	6 h	Mostly human
5	670 000	1 500 000	Chemical floculation	1 h	Mostly human

membranes (Millipore, Molsheim, France). Filtrated samples were cultured on m-FC agar (m-FCA) plates (Difco, Detroit, USA) at 44·5°C for 24 h to enumerate faecal coliforms. Enumeration of blue colonies was carried out at 24 h (Grabow 1990). In addition, another three sets of membranes with filtrated samples were preincubated on brain–heart infusion agar (BHIA) (Difco) at 37°C for no more than 2 h for the recovery of stressed enterococci (Anon. 1998). Membranes were then transferred onto m-*Enterococcus* agar plates (MEA) (Difco), MEA with 8 mg l^{-1} of erythromycin (8E) (Sigma-Aldrich, Saint Quentin Fallveir, France) and MEA with 8 mg l^{-1} of vancomycin (8V) (Sigma-Aldrich), for the enumeration of total enterococci, total enterococci resistant to erythromycin and to vancomycin, respectively. Plates were incubated at 37°C for 48 h. They were then 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. This process is confirmed in this medium by the black colour adopted by the colonies. Black colony counts were then undertaken for each of the membranes analysed to confirm enterococci enumerations (Figueras *et al.* 1998; Manero and Blanch 1999).

Spores of SRB were enumerated by the heat shock of samples at 80°C for 10 min (Handford 1974). Later, 10-fold dilutions were made in Ringer 1/4, and 1 ml of each dilution was inoculated in 50 ml of liquid sulphite polymyxin sulphadiazine (SPS) agar (Scharlau, Barcelona, Spain). Inoculated tubes were shaken to homogenize the solution and to allow the media to solidify. These tubes were then incubated at 44°C for 24 h.

Additionally, because of the low concentration of VRE strains that was assumed, enrichment in Enterococcosel™ broth (Becton Dickinson, Cockeysville, MD, USA) was performed. Aliquots of 10 ml of each sample were inoculated to double concentrated Enterococcosel™ broth containing 16 mg l^{-1} of vancomycin. To isolate strains, aliquots of 10 μl from the 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.

Biochemical fingerprinting

A maximum of 24 statistically representative colonies of each bacterial group (faecal coliforms and enterococci) were randomly isolated from plates containing between 30 and 100 colonies for each sample (Bianchi and Bianchi 1982; Kühn *et al.* 1997). In addition, up to 10 colonies per sample from the 8E plates and 8V plates were also isolated. Vancomycin-resistant strains were isolated from direct plating on 8V plates or from the enrichment broth. Overnight cultures of enterococci and faecal coliforms isolates on BHIA (Difco) were prepared at 37 and 44·5°C, respectively. Cell suspensions were prepared by harvesting these cultures in a suspending medium (w/v): 0·2% proteose peptone (Difco), 0·05% yeast extract (Scharlau, Barcelona, Spain) 0·5% NaCl and 0·011% bromothymol blue (Merck, Darmstadt, Germany) for enterococci, and 0·1% proteose peptone and 0·011% bromothymol blue for faecal coliforms. These cell suspensions were made in the first well of each row of the PhP-RF and PhP-RE microplates (PhP-Plate Microplates Techniques AB, Stockholm, Sweden), 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 by Kühn and Möllby (1993).

The PhP-RF and PhP-RE plates consisted of 96-well microplates containing dehydrated reagents, which were selected to provide a high level of discrimination of populations within enterococci or faecal coliforms, respectively (Kühn *et al.* 1991). The biochemical fingerprinting procedure with these microplates has been described previously by 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 absorbance readings were taken at 16, 40 and 64 h for enterococci, and at 7, 24 and 48 h for faecal coliforms. The biochemical profiles were calculated using accumulative absorbance values as previously described by Kühn *et al.* (1991).

Indices of population diversity, and similarity and clustering analysis

Simpson's diversity index (Di) was used to calculate the diversity of the bacterial populations in each group (Atlas 1984; Hunter and Gaston 1988), while the similarity between populations was calculated using the coefficient of population similarity (Sp) (Kühn *et al.* 1991). The diversity indexes were calculated using the correlation coefficients for the analysed tests and by taking into consideration all the isolates of faecal coliform and enterococci at each point sampled. Comparison of these bacterial populations at the various sampling sites was analysed using the unweighted-pair groups method (UPGM) with average linkage and the clustering of correlations and Sp coefficients (Sneath and Sokal 1973; Kühn *et al.* 1991). ERE and VRE populations were analysed in the same way. The reading, calculations of diversity and population similarity indexes, correlation coefficients and clustering analysis were performed using the Phpwin Software (PhP-Plate Microplates Techniques) as previously described by Kühn *et al.* (1991).

Identification of cluster representative isolates for the calculation of species distribution

The isolates showed distinct clusters (clonal populations) 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. Enterococcal isolates were identified by comparing the biochemical PhP-profiles obtained in the present study with those of 178 representative isolates of almost 20 000 enterococcal isolates performed in an international research project (Kühn *et al.* 2000) at a correlation coefficient higher than 0·965. These 178 isolates were selected as being representative in a previous study because they showed the highest minimum similarity to enterococcal isolates other than those belonging to the same cluster, and the highest mean similarity to all the isolates belonging to the same cluster (Kühn *et al.* 1991). The species identification of all these 178 representative strains was performed by standard methods and the *Enterococcus* matrix is described elsewhere (Manero and Blanch 1999), using Bacterial Identifier software (Blackwell Science Publishers Ltd, Oxford, UK) as recently described (Blanch *et al.* 2003). The percentage of *Enterococcus* species for each sewage sample analysed was calculated by counting the isolates included in clusters belonging to a species. However, enterococci clusters belonging to phylogenetically related species (Behr *et al.* 2000) such as *Enterococcus hirae* and *Enterococcus durans* (H–D group) or *Enterococcus gallinarum*, *Enterococcus casseliflavus* and *Enterococcus flavescens* (C–G–F group) were considered as a unique group. Those not identified were named 'others'.

Considering the high degree of diversity of faecal coliforms observed in previous studies (McLellan *et al.* 2001; Vilanova *et al.* 2002), a clustering analysis with all the faecal coliform isolates was performed. Clusters including more than 1% of the total number of isolates were identified by selecting a representative strain according to the criteria explained above for enterococci. All representative strains of the selected clusters for each sample were stored at −70°C. The representative strains of faecal coliforms were identified using the API 20E system, following the manufacturer's instructions and database profiling (bioMérieux, La Balme, France). Later, the percentages of isolates included in clusters that belonged to the same species provided the species distribution in the populations under analysis.

RESULTS

Enumeration of bacterial populations

The treatment plants with the highest hydraulic retention times (plants 1, 2 and 3) showed a die-off rate of almost 2-log colony counts for the bacterial indicators analysed. In contrast, those with the lowest hydraulic retention times (plants 4 and 5) showed a lower die-off rate that was never > 1-log count (Table 2). The proportion of counts for enterococci and faecal coliform populations after treatment was the same in all the plants studied. However, the reduction of spores of clostridia in plants with the highest retention times was lower ($P < 0{\cdot}05$) than that of faecal coliforms or enterococci, while no differences in the reduction of spores of clostridia, faecal coliforms or enterococci in the plants with the lowest retention times were observed (Table 2). The counts for ERE in the five plants studied were always around 1 : 10 of the total

Table 2 Average log (CFU 100 ml^{-1}) (load) and population diversity (Di) of the bacterial groups analysed in the raw (RS) and treated sewage (TS) from five plants

		FC		Ent		Ery- Ent		Van-Ent	
	Cl load	Load	Di	Load	Di	Load	Di	Load	Di
Plant 1 RS	5·45 (±0·32)	7·47 (±0·45)	0·975 (56)	6·53 (±0·40)	0·942 (119)	5·34 (±0·30)	0·933 (15)	n.d.	0·985 (12)
Plant 1 TS	3·88 (±0·30)	5·09 (±0·77)	0·982 (56)	4·14 (±0·38)	0·974 (102)	2·94 (±0·49)	0·868 (17)	n.d.	0·927 (11)
Plant 2 RS	5·57 (±0·28)	7·26 (±0·28)	0·987 (192)	5·99 (±0·28)	0·944 (266)	5·03 (±0·42)	0·924 (47)	2·12 (±0·57)	0·960 (28)
Plant 2 TS	3·59 (±0·44)	4·53 (±0·56)	0·992 (156)	3·33 (±0·45)	0·963 (166)	2·26 (±0·55)	0·894 (28)	n.d.	0·867 (6)
Plant 3 RS	4·28 (±0·39)	6·09 (±0·38)	0·983 (216)	5·19 (±0·30)	0·956 (200)	4·19 (±0·42)	0·937 (41)	2·10 (±0·28)	0·879 (42)
Plant 3 TS	3·04 (±0·45)	3·84 (±0·34)	0·987 (206)	3·01 (±0·34)	0·947 (235)	2·06 (±0·42)	0·927 (41)	n.d.	0·771 (35)
Plant 4 RS	5·02 (±0·54)	7·29 (±0·24)	0·992 (209)	5·89 (±0·43)	0·949 (227)	4·95 (±0·22)	0·946 (36)	2·28 (±0·48)	0·868 (48)
Plant 4 TS	4·05 (±0·31)	6·47 (±0·47)	0·989 (208)	5·11 (±0·40)	0·952 (234)	4·14 (±0·44)	0·938 (34)	1·91 (±0·21)	0·900 (51)
Plant 5 RS	5·11 (±0·48)	7·10 (±0·35)	0·988 (208)	5·90 (±0·29)	0·920 (185)	5·06 (±0·29)	0·945 (33)	2·49 (±0·43)	0·310 (21)
Plant 5 TS	4·66 (±0·28)	6·60 (±0·46)	0·988 (238)	5·41 (±0·24)	0·947 (206)	4·64 (±0·20)	0·897 (27)	2·14 (±0·39)	0·917 (9)

Values of standard deviation for colony counts and number of isolates analysed for diversity are indicated in brackets.
Cl, spores of clostridia; FC, faecal coliforms; Ent, enterococci; Ery-Ent, enterococci resistant to 8 mg l^{-1} of erythromycin; Van-Ent, enterococci resistant to 8 mg l^{-1} of vancomycin; n.d., not detected (isolates from enrichment broth are not considered in this table).

enterococci counts. VRE were detected at very low proportions, around 1 : 5000 of the total enterococci counts.

Diversity index and population similarities between sampling sites

A total of 1745 faecal coliforms and 2522 enterococci isolates were typed using the PhP-RE and PhP-RF plates, respectively. High diversity index values were found for both bacterial groups at all of the sampling sites (Table 2). The population similarity indexes between raw and treated sewages were high for both bacterial groups (Fig. 1). However, the lowest Sp values were recorded in plant 1 (Fig. 1). Furthermore, the highest similarity indexes were recorded by enterococci populations (Sp values between 0·278 and 0·575 compared with values between 0·075 and 0·351 for faecal coliform populations).

Moreover, high similarity values between raw and treated sewages from all five plants were recorded for the ERE populations. The comparison of VRE populations between sampling sites was conducted by including isolates from the enrichment broth because of the low number of VRE isolates obtained using the membrane filtration method. This low number of isolates conditioned the clustering analysis of this subpopulation when comparisons were made with the analyses of the other populations (Fig. 1). A similarity was also recorded between raw and treated sewage VRE populations at the five plants, although the similarity was not as great as that among the enterococci and ERE populations.

Proportions of distinct species within faecal coliforms and enterococci populations

As the diversity of faecal coliforms was high, only the representative isolates of clusters comprising more than 1% of the total isolated strains were identified. A total of 35 isolates were thus identified using API 20E galleries.

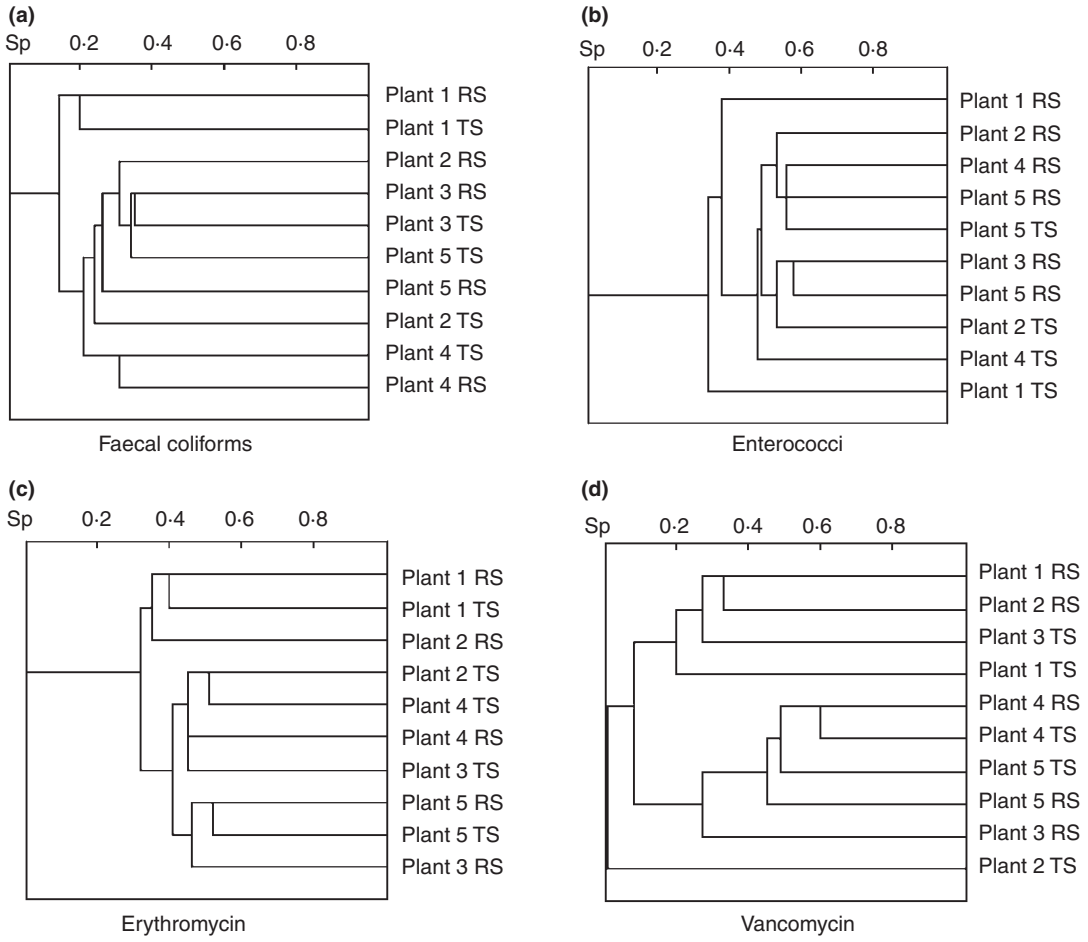

Fig. 1 Dendrograms of the clustering analysis of the similarity of populations (Sp) within each wastewater sampled. (a) Faecal coliform populations, (b) enterococci populations, (c) enterococci resistant to 8 mg l^{-1} of erythromycin populations and (d) enterococci resistant to 8 mg l^{-1} of vancomycin populations (RS, raw sewage; TS, treated sewage)

Table 3 Percentage of the most abundant faecal coliforms in the sewage samples studied

	Classification of faecal coliform isolates (%)								
	E. coli		*Citrobacter*		*Klebsiella*		*Enterobacter*		Others
	API 20E	cpc	API 20E	cpc	API 20E	cpc	API 20E	cpc	cpc
Plant 1 RS	66	(60)	26	(22)	5	(9)	3	(2)	(7)
Plant 1 TS	50	(28)	50	(34)	0	(13)	0	(2)	(23)
Plant 2 RS	78	(55)	20	(16)	1	(7)	1	(3)	(19)
Plant 2 TS	62	(44)	27	(15)	2	(20)	9	(6)	(13)
Plant 3 RS	69	(50)	26	(21)	1	(12)	4	(2)	(15)
Plant 3 TS	57	(44)	36	(28)	0	(10)	7	(4)	(14)
Plant 4 RS	49	(20)	25	(20)	21	(41)	5	(1)	(18)
Plant 4 TS	56	(35)	33	(23)	7	(27)	4	(2)	(13)
Plant 5 RS	65	(41)	33	(23)	0	(19)	2	(3)	(14)
Plant 5 TS	63	(43)	28	(25)	3	(14)	6	(4)	(14)

API 20E: percentage calculated by the identification of representative isolates with API 20E galleries. This percentage refers to the total number of faecal coliforms identified (unidentified profiles are not included).
cpc: percentage calculated from the common phenotypic characteristics obtained by PhP-fingerprinting (*E. coli*: D-(−)-galactono-1,4-lactone (+) cellobiose (–); *Citrobacter*: D-(−)-galactono-1,4-lactone (–) deoxyribose (+); *Enterobacter*: D-(−)-galactono-1,4-lactone (+) cellobiose (+) ornitine (+); *Klebseilla* (2 biochemical profiles): (a) D-(−)-galactono-1,4-lactone (+) cellobiose (+) ornitine (–) (b) D-(−)-galactono-1,4-lactone (–) deoxyribose (–) ornitine (–); Others: other profiles not classified. RS: raw sewage; TS: treated sewage.

Twenty-one of these were classified as *Escherichia coli*, seven as *Citrobacter freundii*, two as *Citrobacter* spp., one as *Klebsiella pneumoniae*, two as *Klebsiella* spp., one as *Enterobacter clocae* and one as *Enterobacter* sp. The identification of these representative strains of clonal population, as defined by the clustering analysis, allowed the subsequent identification of 595 faecal coliform isolates. The common phenotypical characteristics obtained on the biochemical PhP-profiles for the isolates identified as *E. coli* were fermentation of D-(−)-galactono-1,4-lactone (mean of absorbance values ≤ 12) and weak or negative fermentation for cellobiose test (mean of absorbance values >12). All isolates identified as *Citrobacter* presented weak or negative fermentation for D-(−)-galactono-1,4-lactone and positive reaction for deoxyribose test. Isolates identified as pertaining to *Enterobacter* showed fermentation of D-(−)-galactono-1,4-lactone, cellobiose and positive ornitine tests. The biochemical PhP-profiles belonging to clusters whose representative strain did not fit any API 20E profiling showed weak or negative reaction for fermentation of D-(−)-galactono-1,4-lactone and for D-Oxiribose tests. Only one representative strain, identified as *Klebsiella pneumoniae* by API 20E, shared these biochemical properties. Considering these phenotypical characteristics, the correlation coefficients between all the faecal coliforms, the clustering analysis and the identification provided by the API 20E system, the percentages of faecal coliforms (almost 600 isolates) belonging to a cluster (clonal populations) were calculated (Table 3). Isolates presenting a single biochemical PhP-profile were not included as they were not identified by the procedure based on clustering analysis (see above description). The percentage of single biochemical PhP-profiles in each sample typically ranged between 50 and 90% of the total faecal coliforms, although in some cases it reached 100%. These percentages of single isolates are consistent with the high diversity observed in all sewage samples.

There were a number of predominant species or genus among the clonal populations. The most frequent faecal coliform populations belonged to *E coli*, followed by those of the genus *Citrobacter* and, then, *Klebsiella* and *Enterobacter* (Table 3). However, the percentages of *Klebsiella* spp. in the raw and treated sewage from plant 4 were higher than those in the sewage samples from the other plants. Although variations in the percentage of *E. coli*, *Citrobacter*, *Enterobacter* and *Klebsiella* in the clonal populations were observed in some samples after treatment, there were no significant changes in either the composition or structure of these populations as shown by clustering analysis and similarity population indexes.

A total of 1992 enterococcal isolates (79%) belonging to clonal populations were identified by the clustering analysis and identification of representative strains. More than 55% of isolates in each analysed sewage sample belonged to *Enterococcus faecalis* and *Enterococcus faecium* clusters (Table 4) and these were the predominant species in all

Table 4 Percentage of the most abundant enterococci in the clonal populations of the sewage samples studied, based on the identification of representative strains from the clustering analysis

	Classification of enterococci isolates (%)				
	Ent. faecalis	*Ent. faecium*	H–D group	C–G–F group	Others
Plant 1 RS	50	21	12	6	11
Plant 1 TS	29	34	9	13	15
Plant 2 RS	37	38	7	5	13
Plant 2 TS	26	38	12	3	21
Plant 3 RS	26	30	19	5	20
Plant 3 TS	29	36	16	4	15
Plant 4 RS	35	32	16	2	15
Plant 4 TS	47	30	7	5	11
Plant 5 RS	37	33	12	6	12
Plant 5 TS	31	43	5	4	17

H–D group, *Enterococcus hirae* and *Enterococcus durans* group; C–G–F group, *Enterococcus casseliflavus*, *Enterococcus gallinarum* and *Enterococcus flavescens* group.
RS, raw sewage; TS, treated sewage.

cases. VRE isolates (73%) from clonal populations were classified as *Enterococcus faecium*, 10% as belonging to the C–F–G group, 4·5% as *Enterococcus hirae* and 4% as *Enterococcus faecalis*. However, 52% of ERE isolates presented biochemical PhP-profiles of clonal populations classified as *Enterococcus faecalis*, followed by *Enterococcus faecium* (38%) and *Enterococcus hirae* (6%). Clustering analysis and similarity population indexes showed there to be no significant modification in the composition and structure of these clonal populations, although a moderate reduction, or even slight increase, in the percentage of the different groups of *Enterococcus* species was observed in some samples after treatment.

DISCUSSION

The proportions of enterococci, faecal coliform and resistant enterococci were similar in the five treatment plants, both in raw and treated sewage, independent of the treatment plant, the die-off rate (microbial load reduction), and the origin of wastewater. This finding contrasts with the higher persistence of enterococci described in previous studies (Fujioka *et al.* 1981; Scheuerman *et al.* 1987; Barcina *et al.* 1990; Sinton *et al.* 1994; Tree *et al.* 2003). However, it agrees with the observations of other authors who found similar proportions of enterococci and faecal coliforms before and after various treatment procedures (Hill and Sobsey 1998; Payment *et al.* 2001).

Smaller reductions in the spores of clostridia as in those of faecal coliforms or enterococci were found in plants with high retention times (plants 1, 2 and 3). A similar situation has been observed in natural surface waters and treatment systems for swine wastewater (Medema *et al.* 1997; Hill and Sobsey 1998). In contrast, no differences in reduction were observed for these bacterial populations in plants with low retention times (plants 4 and 5). This can be explained by the higher persistence of the spores of sulphite-reducing bacteria (Scheuerman *et al.* 1987; Barcina *et al.* 1990; Sinton *et al.* 1994; Mocé-Llivina *et al.* 2003; Tree *et al.* 2003) or it might be a result of the fact that the short retention time is not sufficient to allow the sporulation of clostridia in the presence of the low availability of oxygen. In contrast, a long retention time with intervals of high aeration might induce the sporulation of clostridia.

Vancomycin- and erythromycin-resistant strains accounted for 0·05 and 10%, respectively, of the total enterococci in all the raw sewage samples analysed, independently of their origin. The die-off rate for VRE and ERE populations in all the analysed plants was similar to the die-off rate for the total enterococci in the corresponding plant. Thus, there are usually no differential factors that promote a higher persistence or reduction of resistant strains. Again, this finding contrasts with that reported elsewhere in which a higher proportion of resistant stains of faecal coliforms (Mezrioui and Baleux 1994) or total coliforms (Andersen 1993) were observed in treated sewage.

A high diversity of both bacterial groups and ERE populations was found in all the sewage samples analysed independently of the treatment plant. VRE isolates also showed a high diversity in many sewage samples but the proportion of these isolates was low and their isolation required prior enrichment which could have conditioned their diversity index. The great similarity between bacterial populations in raw and treated sewage in all the plants studied also indicates the absence of the selective elimination of the faecal coliform and enterococci populations studied as a group. Moreover, high similarity index values between different plants indicate that faecal coliforms and enterococcal populations have similar structures and compositions in distinct types of sewage. This result agrees with previous observations made in an international study in which the sewage from various countries was compared showing a high similarity between urban sewage samples independently of their geographical origin (Blanch *et al.* 2003). The higher diversity and lower similarity indexes of faecal coliforms could be explained by the fact that this bacterial group includes different genus, while that of enterococci is constituted only by the genus *Enterococcus*. These findings are consistent with the high diversity of faecal coliforms, in particular that of the *E. coli* population, detected by other authors (McLellan *et al.* 2001) using pulsed-field gel electrophoresis (PFGE). The latter authors suggest the wide range of profiles obtained for *E. coli* isolates is to be expected from faeces-contaminated water in the absence of

replication in the environment. This, indeed, is the situation we found in our samples. High diversity is not exclusive to environmental *E. coli* isolates. It has also been described for clinical isolates in epidemiological and outbreak studies (Rice *et al.* 1999; Heir *et al.* 2000).

In spite of this high similarity, plant 1, which processes wastewater of mixed origin (human and animal), showed the lowest similarity with the others, whose wastewater was mostly of human origin. Similar observations have been made when comparing hospital sewage with pig slurries (Blanch *et al.* 2003). However, widespread studies of the biochemical fingerprinting of bacterial populations in wastewaters of different faecal origins are needed to determine specific and worldwide phenotypes related to the source of pollution.

The distribution of enterococci species observed is in agreement with the findings of previous studies (Sinton and Donnison 1994; Laukova and Juris 1997; Svec and Sedlacek 1999) that show that *Enterococcus faecium* and *Enterococcus faecalis* are the most prevalent species in all sewage treatment plants. The different sewage composition of plant 1 did not affect its enterococcal distribution. The results from some sewage samples might indicate that certain enterococcal species (*Enterococcus faecalis* and *Enterococcus hirae – Enterococcus durans* group) are eliminated at higher rates than others (*Enterococcus faecium* and *Enterococcus casseliflavus – Enterococcus gallinarum – Enterococcus flavescens* group). However, our study does not allow us to draw this conclusion because first a specific enumeration and calculation of die-off rates for each species would have to be performed. In this study, species percentages were calculated by clustering analysis and, subsequently, by retrospective calculation of the isolates. The distribution of faecal coliform species is in agreement with previous studies (Brown and Tracey 1975; Mersch-Sundermann and Wundt 1987; Hill and Sobsey 1998; McLellan *et al.* 2001). Four main groups of faecal coliforms (*E. coli*, *Citrobacter* spp., *Klebsiella* spp. and *Enterobacter* spp.) were found among the clonal populations defined by clustering analysis. A high percentage of faecal coliform isolates presented single biochemical PhP-profiles. This situation has also been observed elsewhere using other fingerprinting (PFGE) approaches (McLellan *et al.* 2001). The latter also characterized some of these isolates using the API 20E system and determined most of them to be *E. coli*. The high diversity of profiles among *E. coli*, and perhaps other faecal coliforms, is due to the fact that faecal pollution in sewage generally originates from hundreds or thousands of hosts. However, it has also been shown that sewage from a population of 5000 people provides similar faecal coliform and enterococci populations to treatment plants receiving sewage from hundreds of thousands humans. The suggested reduction in the percentage of *E. coli* and *Klebsiella* spp. and the lower reduction or even increase in the percentage of *Citrobacter* and *Enterobacter* in some treated sewage samples could support findings reported elsewhere in which the clonal populations of faecal coliforms other than *E. coli* provided evidence of the possible replication of those other organisms in the environment. Were this to be the case, it would support the use of *E. coli* as an indicator for the analysis of faecal pollution in waters rather than the use of faecal coliforms (McLellan *et al.* 2001).

The persistence of antibiotic resistant bacteria in sewage observed in this study suggests there is no selective elimination of bacterial populations in the treatment process. The ability of antibiotic resistant strains to survive sewage treatment systems should be considered in certain water reuse programmes (Anderson 2001) especially if treated sewage can be used in high contact areas (agricultural application) and those open to human population (recreational facilities). In this way the transference and persistence of antibiotic resistant genes could be facilitated.

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Capítol 2

The effect of a sewage treatment plant effluent on the coliforms and enterococci population of the reception river waters

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RESUM DEL CAPÍTOL

En aquest cas, i aplicant la mateixa metodologia que en el capítol anterior, es van comparar l'estructura i la composició de les poblacions de coliforms fecals i enterococs presents en les aigües residuals crues i tractades d'una depuradora, amb les de les aigües del riu on s'abocaven aquestes darreres.

Els resultats obtinguts mostraren una elevada similitud tant entre les poblacions de coliforms fecals com en les d'enterococs dels diferents tipus de mostra analitzades. Les mostres d'aigua residual tractada mostraren una reducció aproximada de 2 logaritmes en el cas de coliforms fecals i enterococs. Per la seva banda, l'aigua tractada presentava una càrrega molt semblant a l'aigua del riu en els diferents punts de mostreig. Tot i que no es va presentar a l'article, en aquest treball també es van analitzar els enterococs resistents a l'eritromicina i a la vancomicina així com les espores de clostridis sulfit-reductor, obtenint els resultats resumits en la taula 3 i que es comenten en la discussió d'aquesta memòria.

Taula 3. Mitjana dels valors logarítmics d' UFC/100ml d'espores de clostridis sulfit-reductors (SRCS); Enterococs resistents a l'eritromicina (ERE); enterococs resistents a la vancomicina (VRE).

	SCRS	**ERE**	**VRE**
Riu amunt (P0)	2,79(± 0,20)	1,8 (±0,64)	<1
Aigua residual (E)	4,28 (± 0,39)	4,19 (±0,42)	2,10 (±0,28)
Aigua tractada (S)	3,04 (± 0,45)	2,06 (±0,42)	<1
50m riu avall (P1)	2,86 (±0,18)	2,02 (±0,49)	<1
2000m riu avall (P2)	2,77 (±16)	1,86 (±0,44)	<1

Nota: Tant en les aigües tractades com en les aigües de riu només van ser detectats enterococs VRE en els enriquiments, per la qual cosa la concentració era menor a 10 cfu/100 ml. (<1 en valors logarítmics).

La conclusió d'aquest capítol fou que les emissions de l'aigua tractada a la depuradora no afectaven a les poblacions d'enterococs i coliforms fecals de les aigües del riu que les rebia. A més a més, la reducció de la càrrega microbiana de les aigües riu avall, no fou significativa.

APORTACIÓ PERSONAL AL TREBALL

L'autor d'aquesta tesi ha realitzat el disseny del mostreig, la presa de mostres, les anàlisis microbiològiques i l'anàlisi del resultats obtinguts amb la col·laboració del Dr. Albert Manero Camps en la identificació de les soques d'enterococs i de la Dra. Marta Cerdà Cuéllar en el Phene-System, així com la supervisió del professor Anicet R. Blanch Gisbert.

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The effect of a sewage treatment plant effluent on the faecal coliforms and enterococci populations of the reception river waters

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Aims: A rural sewage treatment plant and the effect of its effluent on the enterococci and faecal coliforms populations of the receiving river waters was evaluated.

Methods and Results: The enumeration of bacteria was performed by membrane filtration. Diversity and population similarity were analysed using the PhP-plates system. The treatment plant reduces the number of enterococci and faecal coliforms to values similar to those observed upstream. All water samples showed a high diversity for both bacterial populations. A high similarity in the composition and structure, was detected among all the samples.

Conclusions: The impact of the disposal of treated sewage on the river did not modify the composition of either bacterial populations in the river water.

Significance and Impact of the Study: The biochemical phenotyping of bacterial populations is a reliable tool for ecological and biodiversity studies. The obtained results provide a better understanding of the sewage treatment process and the impact of the treated sewage effluents in the environment.

INTRODUCTION

Faecal coliforms and enterococci have been used widely as faecal pollution indicators (Sinton *et al*. 1998). Both microbial groups can be determined by their enumeration, the proportion of faecal coliforms/enterococci or their inactivation by different agents. Both bacterial groups include several species. For example, the genus *Enterococcus* contains 19 recognized species (Manero and Blanch 1999). Any determination of their diversity in the environment should consider this aspect.

Urban or rural waste waters normally contain many bacterial species, each with a large number of strains. Biological treatment processes at sewage treatment plants could produce selective elimination, changes of proportion or both in the bacterial populations (Mezrioui and Baleux 1994). Moreover, the sewage treatment plant effluent, as well as urban or industrial waste, could modify some microbial populations in the reception waters, such as rivers, lakes or lagoons (Sinton and Donnison 1994; Kühn *et al*. 1997). This effect could become more important where policies of water re-utilization are applied in regions with poor water resources. The determination of the origin of faecal pollution in waters is important for the management and quality control of water resources. Subtyping below the species level of bacteria could provide valuable information about the sources of pollution in surface waters.

In this study, the effect of an effluent from a rural sewage treatment plant on enterococci and faecal coliforms populations of the reception river waters was evaluated. Both bacterial groups were enumerated. The biodiversity and the similarity of the composition and structure of their populations was also studied.

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MATERIALS AND METHODS

Sampling, pre-treatment and enumeration of bacterial populations

The studied plant treated 7500 m^3 day^{-1} of sewage using an activated sludge procedure. The studied river has a typical Mediterranean irregular flow. The treated sewage volume represents a 15·8% of the lowest annual river flow, a 0·05% of the highest annual flow and 0·9% of the annual average river

flow (Anonymous 1990). The nearest upstream treatment plant was located at 7 km, and no other controlled faecal pollution effluent was presented. Five sampling sites were defined for their analysis: raw sewage (W1), treated sewage effluent (W2), 50 m upstream water (W0), 50 m downstream water (P1) and 2 km downstream water (P2). A minimum of six samples was taken from each point on different days for a 2-year period. Samples were stored, transported and kept at 4°C for less than 6 h until they were processed. The enumeration of faecal coliforms and enterococci was performed by membrane filtration through 0·45 μm pore size membranes (Millipore). Filtered samples were pre-incubated on brain heart infusion agar (BHIA; Difco) at 37 °C for 2 h for the enumeration of enterococci. This enrichment allowed the recovery of stressed cells. Later, membranes were transferred sequentially onto m-*Enterococcus* agar plates (MEA; Difco) and incubated at 37 °C for 48 h. Colony counting of enterococci was performed by sequentially transferring membranes from MEA onto Bile Esculine Agar (BEA; Difco) for 1 h at 44 °C. Hydrolysis of esculine was manifested by black colouration for the enterococci colonies. (Figueras *et al*. 1998; Manero and Blanch 1999). At the same time, another set of membranes with filtered samples were prepared for faecal coliform enumeration. The membranes were placed on m-FC agar plates (mFCA; Difco) at 44 °C for 24 h. The counts of blue colonies provided the enumeration of this population (Grabow 1990). Analysis of variance was used in order to determine the significance of the results, with statistical software (Statgraphics, Statistical Graphics Corporation).

Biochemical fingerprinting

A maximum of 24 colonies was isolated randomly for each sample and bacterial group. They represented the faecal coliforms and enterococci populations associated with each sample according to Bianchi and Bianchi (1982) and Kühn *et al*. (1997). Overnight cultures on BHIA were prepared at 37 °C and 44 °C for enterococci and faecal coliform isolates, respectively. Cell suspensions were prepared by harvesting overnight cultures in a solution of distilled water at 0·2% w/v proteose peptone, 0·05% w/v yeast extract, 0·5% w/v NaCl and at 0·011% w/v bromothymol blue for enterococci, and 0·1% w/v proteose peptone (Difco) and 0·011% w/v bromothymol blue for faecal coliforms. These cell suspensions were used for inoculation of PhP-plates (PhP-Plate Microplates Techniques AB, Sweden) according to the manufacturer's instructions and as described previously (Kühn and Möllby 1993). The PhP-RS and PhP-RE plates consist of 96-well microplates with dehydrated reagents, which have been selected to provide a high level of discrimination among enterococci and faecal coliforms, respectively. The biochemical fingerprinting procedure with these PhP-plates has been described earlier (Kühn 1985). Aliquots of 25 μl of bacterial suspension were added to all the wells of the same row of a microtitre PhP-RS plate for enterococci isolates. Faecal coliform isolates were analysed by the same protocol but with a PhP-RE plate. The inoculated microtitre plates were incubated at 37 °C for both bacterial groups. The growth in the different wells was measured in a spectrophotometer at 620 nm with the iEMS Reader MF (Labsystems, Helsinki, Finland). These readings were performed at 16 h, 40 h and 64 h for enterococci, and at 7 h, 24 h and 48 h for faecal coliforms. The biochemical profiles were calculated according to Kühn *et al*. (1991).

Diversity index and population similarities in different samples

Simpson's diversity index (Di) and the population similarity index (Sp) were calculated according to Kühn *et al*. (1991) with the PhP Software (PhP-Plate Microplates and Techniques AB, Sweden). The diversity index (Di) was calculated for each bacterial group and for each sample. Average diversity of the different samples from each sampling site were also calculated. The pairwise comparison between all samples was calculated. Averages of similarity indexes within each sampling site were calculated, and cluster analysis for each site was performed. Moreover, the comparison of samples was also performed for each sampling occasion, calculating similarity indexes and cluster analysis. The average of the similarity indexes for each sampling occasion was also determined.

Diversity index and population similarity in pooled isolates of each sampling site

Isolates from each sampling site were pooled, providing over 150 isolates for each site and bacterial group. The diversity of pooled populations and their population similarity were also calculated. Differences between the diversity index of pooled isolates and the average of diversity indexes of samples in each site provided information about regularity of microbiota constitution in each sampling site. Consequently, a site with a low average value of diversity and a high value of diversity for pooled samples would indicate that the high diversity is due to a succession of differing but homogeneous populations. In this case, a low value of pairwise population similarity between samples of that sampling site should be obtained.

RESULTS

Enumeration of bacterial populations

Treated sewage showed a 2-log reduction of the value for the enumeration of both populations with respect to raw

sewage. Also, counts for both bacterial populations were 2-log lower in upstream and downstream samples when compared to raw sewage.

Diversity index and population similarities in different samples

A total number of 1227 faecal coliforms and 1248 enterococci isolates were typed by the PhP-RE and PhP-RF plate systems, respectively. Their biochemical profiles were calculated and were used for the analysis on the structure of populations. The diversity index of faecal coliforms and enterococci from individual samples indicated a high diversity for both bacterial groups. However, the diversity was always higher for faecal coliforms than for enterococci (Table 1). A higher diversity in enterococci populations from treated sewage was observed with respect to up- and downstream and raw sewage samples. However, statistically significant values of diversity were obtained only for upstream and raw sewage ($P < 0{\cdot}05$). The pairwise comparison of all the samples showed a high similarity for both studied populations. However, enterococci populations showed higher values of similarity (average of Sp = 0·29) than faecal coliforms populations did (average of Sp = 0·10) based on similarity indexes of pairwise studies. Analysis within each group showed that the treated sewage presented the highest similarity (averages of Sp = 0·39 for enterococci and Sp = 0·14 for faecal coliforms) with respect to the other sampling sites. Samples of the different sites when compared by sampling occasion were similar. The average of Sp in the different sampling occasions ranged between 0·22 and 0·39 for enterococci and between 0·082 and 0·18 for faecal coliforms. Cluster analysis differentiated two groups of water samples: upstream, 50 m and 2000 m downstream samples (Sp average = 0·24 and 0·44 for faecal coliforms and enterococci, respectively), and raw and treated sewage (Sp average = 0·15 and 0·36 for faecal coliforms and enterococci, respectively).

Diversity index and population similarity in pooled isolates of each sampling site

The diversity index (Di) calculated with the pooled isolates of each sampling site (Table 1) was higher than the average of the diversity of samples, for any studied bacterial group. Moreover, enterococci populations showed higher values of similarity than faecal coliforms did at different sites. The population similarity study of pooled samples showed that water from upstream and downstream sampling sites presented higher similarity for enterococci and faecal coliforms population than did sewage and treated sewage (Fig. 1).

DISCUSSION

The concentration of faecal coliforms and enterococci populations were similar in almost all the sampling points of the river. Raw sewage always had a higher bacterial concentration for both groups studied. There is a 2-log reduction of the enumeration values from raw to treated sewage. This regular reduction indicates an adequate working condition for the treatment plant. Such reduction values were in agreement with others observed in similar treatment plants (Bitton 1994). The treated sewage effluent had a similar microbial load to the receiving waters, considering the enumerated bacterial populations. The diversity indexes were always higher for faecal coliforms than for enterococci populations. This could be explained partially by the high number of genus and species included in faecal coliform with respect to enterococci populations. Higher similarity values for enterococci with respect to faecal coliform populations could be explained partially for the same reason.

A higher regularity for enumeration values, diversity and population similarity indexes were observed in the treated sewage. This is supported because there were no differences between the diversity of pooled isolates and the

Table 1 Enumeration and diversity of faecal coliforms and enterococci in each sample and in pooled isolates from each site

	Faecal coliforms			Enterococci		
	Counts* $\log_{10}$ cfu ml^{-1}	$\bar{D}i$†	Di‡	Counts* $\log_{10}$ cfu ml^{-1}	$\bar{D}i$†	Di‡
Upstream (P0)	1·82 (± 0·24)	0·939 (± 0·092)	0·973	0·92 (± 0·58)	0·883 (± 0·063)	0·920
Raw sewage (E)	4·17 (± 0·44)	0·985 (± 0·005)	0·988	3·23 (± 0·34)	0·887 (± 0·094)	0·942
Treated sewage (S)	1·99 (± 0·13)	0·985 (± 0·009)	0·988	1·07 (± 0·19)	0·947 (± 0·014)	0·950
50 m downstream (P1)	1·82 (± 0·23)	0·952 (± 0·067)	0·985	0·89 (± 0·49)	0·898 (± 0·089)	0·930
2000 m downstream (P2)	1·70 (± 0·26)	0·982 (± 0·016)	0·992	0·77 (± 0·34)	0·887 (± 0·074)	0·937

*Average of the log values of cfu ml^{-1}; † Diversity of samples. Average of the diversity indexes for the different samples in each sampling site. Standard deviation in brackets. ‡ Diversity on site: diversity index for pooled isolates of each sampling site.

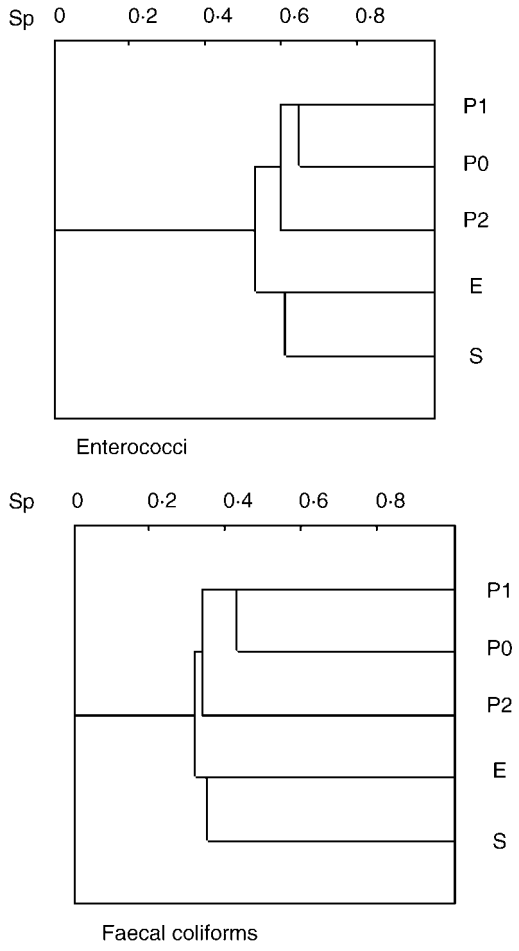

Fig. 1 UPGMA clustering of Sp coefficients obtained from comparisons of bacterial populations in all sampling sites. Each population is constituted by the pooled isolates form all samplings occasions. E: raw sewage; S: Treated sewage; P0 Upstream; P1 50 m downstream; P2 2000 m downstream

average of the diversity by samples in treated sewage. Moreover, there was a higher similarity within treated sewage samples than for the other sites. The activated sludge treatment process produces homogenization of the bacterial population because of the long retention time (over 1 day) and high and continuous stirring. This homogenization could explain the regularity observed in the treated sewage samples.

There is a high similarity between sampling occasions for any of the studied bacterial populations' structure and composition. A high population similarity was also found inside and between all five sampling points. However, cluster analysis showed a higher similarity between the three sampled river points, with respect to the raw and treated sewage samples (Fig. 1). The existence of two different water flows (sewage treatment and river flows) could explain the differentiation of these groups. The high similarity between sewage and downstream to upstream samples could be related to the effluent of a sewage treatment plant 7 km upstream or any uncontrolled input of faecal pollution.

The disposal of studied treated sewage effluent has no effect on the load, the structure or the composition of enterococci and faecal coliforms in the river during the study period. Both bacterial groups presented a homogeneous distribution in the water environments studied, so no selection factor for growth or elimination was acting on these populations.

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Capítol 3

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

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RESUM DEL CAPÍTOL

Una vegada constatada la similitud entre les poblacions de coliforms fecals, enterococs, enterococs resistents a l'eritromicina (ERE) i enterococs resistents a la vancomicina (VRE) de les aigües crues i tractades a les diferents estacions depuradores d'aigües residuals analitzades; vista també la similitud entre les poblacions microbianes esmentades de les aigües tractades d'una de les depuradores analitzades i les de les aigües del riu on abocava l'efluent de la mateixa, es va plantejar la comparació de les poblacions de coliforms fecals, enterococs VRE i ERE aigües residuals d'una depuradora amb les dels fangs produïts en diferents estadis del tractament. Es plantejava si els llots d'una depuradora permetien una presència selectiva d'alguns clons poblacionals dels indicadors bacterians analitzats i si podien ser un reservori de poblacions de VRE i ERE. Això podria facilitar la dispersió de resistències a través de l'abocament o la reutilització dels llots. Els resultats obtinguts, seguint la mateixa metodologia descrita en els dos capítols anteriors, mostraren una elevada similitud entre les poblacions microbianes analitzades. No obstant, aquesta similitud fou menor entre les poblacions dels fangs i la de les aigües residuals crua i tractada. Així mateix, les proporcions entre la càrrega microbiana dels diferents indicadors analitzats fou diferent en els fangs que a les aigües analitzades. VRE i ERE persisteixen també en els llots mantenint una proporció similar amb els enterococs totals a la que presenten en les aigües residuals. Aquesta observació cal considerar-la segons quin sigui el destí final dels llots.

APORTACIÓ PERSONAL AL TREBALL

L'autor d'aquesta tesi ha realitzat el disseny del mostreig, la presa de mostres, les anàlisis microbiològiques i l'anàlisi del resultats obtinguts amb la supervisió del professor Anicet R. Blanch Gisbert.

Category: Full paper

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

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SUMMARY

The changes in composition and structure of faecal 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 enteroccocal populations in two kind of sludge was also considered to evaluate their potential elimination on 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 one log unit higher than ENT and near 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 kind of samples. Antibiotic-resistant enterococci persisted in a similar proportion 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, faecal 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 faecal 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 faecal coliforms or enterococci in river waters and in swine wastewater (Hill and Sobsey, 1998; Medema et al., 1997). Gram negative bacteria are selectively preyed for ciliates (González et al., 1990). It has been also 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 respect to raw sewage. However, it has been reported that in some treatment plants, the proportions of faecal 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 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, different proportion of these bacterial indicators has been reported when comparing sludge respect sewage (Chauret et al., 1999). However, the diversity of FC and ENT populations in sludge has not been analysed.

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 with 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 faecal origin. Then, the reutilization of treated sewage or sludge could carry out 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 faecal coliforms and enterococci in sludge derived form a sewage treatment plant have been analysed. These populations have been compared and related with those in raw and treated sewage. The accumulation and persistence of VRE and ERE in two kind of sludge (liquid and dewatered) have been determined and compared with the rest of 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 were sampled. The plant treats diary 2000 m^3 from a population of 5000 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 recycling sludge channel, with a dry weight residue of 0.4-0.6 g/100ml, and dewatered sludge obtained at the end of the process, with 14-15 g/100ml of dry weight residue. Samples were collected and stored at 4ºC following standard protocols (Anon., 1994). Dewatered sludge samples were homogenizated by magnetic agitation during 30 minutes in a solution of Ringer ¼ in proportion 1:10 w/v, while liquid sludge were homogenizated in the same way without addition of Ringer.

The enumeration of faecal coliforms and enterococci was performed by membrane filtration (Anon., 1997a, 1997b) on 0.45 μm of pore size membranes (Millipore, Molsheim, France). Filtrated samples were cultured on m-FC Agar (mFCA) plates (Difco, Detroit, U.S.A.) at 44.5 ºC for 24h to enumerate faecal coliforms. Counts of blue colonies were done at 24h (Grabow, 1998). In addition, another 3 sets of membranes with filtrated samples were pre-incubated on Brain Heart Infusion Agar (BHIA) (Difco) at 37 ºC for no more than 2h for the recovery of stressed enterococci (Anon., 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, resistant enterococci to erythromycin and to vancomycin respectively as previously described (Blanch et al., 2003). Plates were incubated at 37ºC for 48h. Then, they were transferred to Bile Esculine Agar (BEA) (Difco) for 1h at 44ºC 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^{TM} broth (Becton Dickinson, Cockeysville, MD, USA) was performed in order to isolate resistant strains for the diversity index calculations. Aliquots of 10ml of treated sewage samples were inoculated to double concentrated Enterococcossel^{TM} broth containing 16 mg l^{-1} of vancomycin. To isolate strains, aliquots of 10 µl from tubes showing growth were seeded on 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 (homogenisation 1:10 in the case of sludges) at 80ºC for 10 minutes (Handford, 1974). Later, ten-fold dilutions were made in Ringer ¼, 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 44ºC for 24h.

Biochemical fingerprinting

A maximum of 24 colonies of each bacterial group (faecal coliforms and enterococci) were randomly isolated from plates showing between 30 and 100 colonies for each sample. These colonies represent the faecal coliforms and enterococci associated with each sample (Bianchi and Bianchi, 1982). Overnight cultures of enterococci and faecal coliforms isolates on BHIA were prepared at 37ºC and 44.5ºC 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 faecal 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 others 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-wells microplates containing dehydrated reagents, which have been selected to provide a high level of discrimination of populations within enterococci or faecal coliforms respectively (Kühn et al., 2000; Kühn et al., 1991). The biochemical fingerprinting bases of these microplates has been described previously (Kühn, 1985). Inoculated PhP-RF and PhP-RE microplates were incubated at 37ºC. Growth in wells was measured by using the iEMS Reader MF (Labsystems Helsinki, Findland) at 620 nm. Three readings were performed at 16h, 40h and 64h for enterococci, and at 7h, 24h and 48h for faecal 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 faecal coliform and enterococci for each kind of sample. The comparison of the populations of these bacteria between different kind 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 as described in previous studies respectively (Vilanova et al., 2004; Blanch et al., 2003). 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 gallinarum*, *Enterococcus casseliflavus* and *Enterococcus flavescens* (C-G-F group) were considered as an 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 were taken from each kind of sewage and sludge. The treatment plant showed a reduction of around 2.5 log units of colony counts for faecal 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 1:50000 vancomycin resistant counts respect 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 faecal 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 of kind of samples (Table 1). However, these indexes were always slightly higher for faecal 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 (Figure 1), though enterococci populations showed higher similarity (Sp values between 0.32 and 0.47 vs. 0.15 and 0.25 for faecal

coliform populations). However liquid and dewatered sludge by one side and treated and raw sewage by another side were more similar among them for any of studied bacterial group.

Proportions of distinct species within faecal coliforms and enterococci populations

The most frequent faecal 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).

DISCUSSION

While faecal coliform were the predominant of the studied populations in raw and treated sewage, in both kind of sludge the three studied bacterial groups tended to similar proportions. This situation could be related to the inclusion of bacteria into sludge particles as other authors suggested (Stenstrom and Carlander, 2001). The increase of concentration in dewatered sludge is directly proportional to increase of dry weight. These results are partially in agreement with other authors (Chauret et al., 1999) which 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 to previous studies (Hill and Sobsey, 1998; Payment et al., 2001; Vilanova et al., 2004,). Spores of SRB were found in higher proportion respect to the others 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 ratio ENT / FC 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 respect to raw sewage, whereas decreased in the case of the others bacterial groups. This could be explained because the aeration process, which could induce the sporulation of SRB.

While in treated sewage both faecal coliform and enterococci populations presented similar die-off, enterococci populations are accumulated in higher proportion than faecal 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) these predation affect selectively to gram negative bacteria population, such as faecal coliforms.

ERE strains accounted for 10 % of the total enterococci in all the analysed 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 promote higher persistence or reduction of resistant strains. This finding contrast with that reported elsewhere in which a higher proportion of resistant strains of faecal 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 resistant can persist on the sludge as well as in the treated water. This aspect should be considered when sludge are disposed 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 faecal coliforms. These results are in agreement with previous studies (Brown and Tracey, 1975; Laukova and Juris, 1997; Hill and Sobsey, 1998; McLellan and Salmore, 2001; Sinton and Donnison, 1994; Svec and Sedlacek, 1999; Vilanova et al., 2004). *E. coli* was the main faecal 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. A high diversity indexes for faecal 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 and sludge dewatering processes. 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 each studied bacterial group. Though both bacterial groups showed high similarity of populations in any case, liquid and dewatered sludge were more related between them than to the two kinds of analysed 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 into sludge, where FC have the highest reduction respect the other analysed 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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	Cl	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)

Table 1. Enumeration and diversity of the analysed bacterial populations. **Load**: average of log (CFU x 100 ml^{-1}) for sewage and liquid sewage or log (CFU x 100 gr-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. **Cl** : spores of sulphite-reducing anaerobes (clostridia) **FC**: Faecal 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.

	Faecal 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

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

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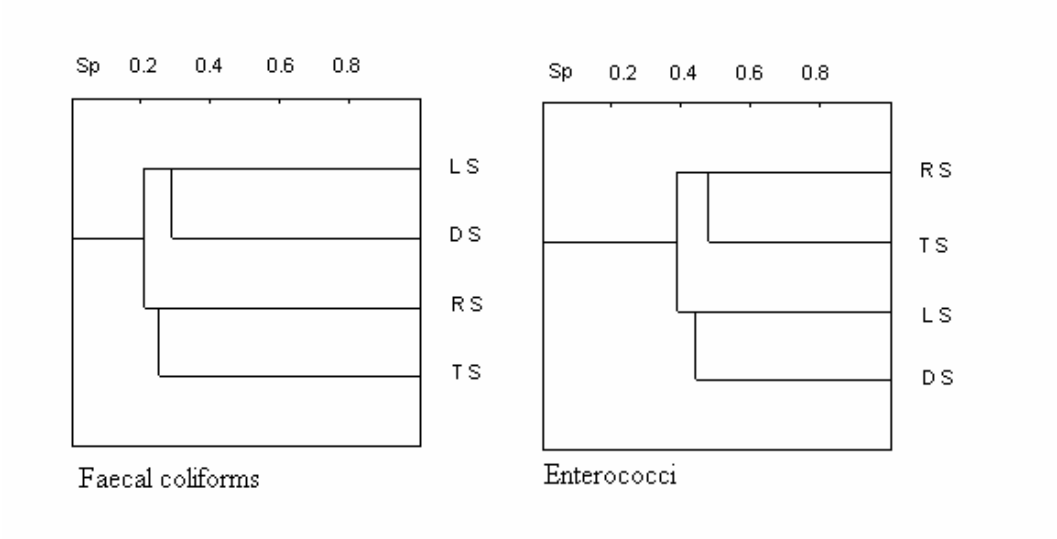

Figure 1. Dendrograms of the clustering analyses based on the similarity of population index (Sp) between the different studied samples. RS: raw sewage; TS: treated sewage; LS: liquid sludge; DS: dewatered sludge.


Greenland
Jan Mayen
Barents Sea
Greenland Sea
Norwegian Sea
Reykjavik
ICELAND
NORWAY
FINLAND
SWEDEN
Tórshavn
Faroe Islands
Helsinki
St. Petersburg
RUSSIA
Oslo
Stockholm
Tallinn
ESTONIA
Moscow
North Atlantic Ocean
North Sea
Riga
LATVIA
LITHUANIA
Vilnius
Minsk
BELARUS
DENMARK
Copenhagen
Dublin
IRELAND
UNITED KINGDOM
London
Amsterdam
Berlin
Warsaw
POLAND
Kiev
UKRAINE
GERMANY
Prague
Paris
Vienna
Budapest
Bratislava
Chisinau
MOLDOVA
ROMANIA
Bucharest
FRANCE
Zagreb
Belgrade
BULGARIA
Sofia
Bay of Biscay
Bilbao
Madrid
Barcelona
PORTUGAL
Lisbon
SPAIN
ITALY
Rome
Tirana
Skopje
GREECE
Athens
TURKEY
Mediterranean Sea
Algiers
Tunis
Valletta
MALTA
Rabat
Casablanca
MOROCCO
ALGERIA
TUNISIA
Scale 1: 19,500,000
Lambert Conformal Conic Projection, standard parallels 40°N and 56°N

Capítol 4

Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions

A. R. Blanch, J. L. Caplin, A. Iversen, I. Kühn, A. Manero, H. D. Taylor, X. Vilanova.

Journal of Applied Microbiology 2003, **94**, 994-1002

RESUM DEL CAPÍTOL

Aquest capítol es va centrar en la comparació de les poblacions d'enterococs, enterococs resistents a la vancomicina (VRE) i enterococs resistents a l'eritromicina (ERE) presents a les aigües residuals municipals i d'hospital, així com també aigües receptores dels efluents de les depuradores, de diferents regions europees. Les aigües residuals municipals eren procedents de diferents tipus de depuradores. Els resultats obtinguts mostraren una elevada similitud entre les poblacions d'enterococs de tots els tipus de mostra analitzats, independentment del país d'origen. També mostraren uns elevats índexs de diversitat poblacional i un predomini de les espècies *Enterococcus faecium* i *Ent. faecalis*. En el cas dels ERE també s'observà una elevada similitud poblacional entre totes les mostres. En el cas dels VRE la similitud poblacional fou baixa, essent en aquest cas clarament dominant l'espècie *Ent. faecium*.

APORTACIÓ PERSONAL AL TREBALL

L'autor d'aquesta tesi ha realitzat el disseny del mostreig, la presa de mostres i les anàlisis microbiològiques en tots aquells punts analitzats situats dintre de l'Estat Espanyol, amb la col·laboració del Dr. Albert Manero Camps en la identificació de les soques d'enterococs i de la Dra. Marta Cerdà Cuéllar en el Phene-System, i sota la direcció i la supervisió del professor Anicet R. Blanch Gisbert.

Journal of Applied Microbiology 2003, **94**, 994–1002

Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions

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ABSTRACT

A.R. BLANCH, J.L. CAPLIN, A. IVERSEN, I. KÜHN, A. MANERO, H.D. TAYLOR AND X. VILANOVA. 2003.

Aims: Scarce knowledge about the distribution of enterococci species in wastewaters limits any statement on their reliability as faecal indicators or the implications of antibiotic resistance transmission by these organisms through the water cycle. Enterococci have been involved in nosocomial infections and the spreading of antibiotic resistance through the food chain. The species distribution of enterococci and the presence of resistant strains to vancomycin and erythromycin were analysed in more than 400 raw and treated urban wastewaters, surface waters receiving these treated wastewaters and hospital wastewaters from three European countries.

Methods and Results: A total of 9296 strains were isolated and biochemically phenotyped. The species identification was based on the comparison of biochemical profiles with those of more than 20 000 enterococci isolates from an international study. The prevalence of enterococcal isolates resistant to erythromycin (ERE) and vancomycin (VRE) was also analysed. ERE strains were present in a high proportion in all the studied samples. VRE strains were also isolated in all studied countries despite the time elapsed since the use of antimicrobial glycopeptides in animal production was banned in the European Union.

Conclusions: *Enterococcus faecalis* and *Ent. faecium* were the most abundant species in all the studied wastewaters. All the studied wastewaters demonstrated high diversity and similar population structure and composition. ERE and VRE isolates were detected in most of the wastewaters.

Significance and Impact of the Study: Urban and hospital wastewaters are useful targets for the evaluation of the prevalence of ERE and VRE isolates in the environment. It appears that these bacteria could pass through wastewater treatment plants and be transferred to surface waters.

Keywords: *Enterococcus*, erythromycin, Spain, Sweden, United Kingdom, vancomycin, wastewater.

INTRODUCTION

Enterococci are present in the gastro-intestinal tract of warm-blooded animals. These bacteria are released to the environment via wastewater. The genus *Enterococcus* is a subgroup of the former faecal streptococci group. Members of the genus may be differentiated from other streptococci by their ability to grow in 6·5% sodium chloride, at pH 9·6, and 10 and 45°C. Enterococci are often used as indicators of microbial quality in waters (Anonymous 1998; Sinton *et al.* 1998). The ratio of this group to faecal coliforms has controversially been proposed as a faecal source indicator (Feachem 1975). The proportion of enterococci to other streptococci in faeces differs among host species. It has been

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reported that there is a predominance of enterococci in human faeces and wastewater, whereas animal sources also contain significant numbers of other streptococci. Such observation and the higher persistence of enterococci in the environment initially supported the view that species identification of enterococci could provide a tool for the determination of faecal pollution sources (Sinton and Donnison 1994). However, this approach has been regarded as unreliable (Anonymous 1998). On the other hand, studies of the distribution and characterization of enterococci species in wastewaters are scarce (Laukova and Juris 1997; Svec and Sedlacek 1999). This lack of knowledge on the variation in species distribution between different wastewaters and geographic areas limits any conclusion as to their reliability as an indicator of faecal source or their implication in the transmission of antibiotic resistance through the water cycle. Though enterococci are not considered primary pathogens, they have been increasingly involved in nosocomial infections worldwide (Linden and Miller 1999). They have also been implicated in the spread of resistance to the glycopeptide antibiotic vancomycin (vancomycin-resistant enterococci, VRE) in hospitals and animal production (Goossens 1998; Teuber and Perreten 2000). The main origin of vancomycin resistance in these environments has been postulated to be the increase of vancomycin usage in human clinical practice (Méndez-Álvarez *et al.* 2000) or the use of the glycopeptide avoparcin as a feed additive in animal husbandry (Bates *et al.,* 1993; Aarestrup 1995). A similar situation has been described for erythromycin resistant enterococci (ERE), which has been related to the use of 16C-macrolide tylosin as a growth promoter (van den Bogaard *et al.,* 1997). Moreover, the persistence of glycopeptide-resistant enterococci has also been suggested to be a consequence of co-selection by the continued use of tylosin for growth promotion (Aarestrup 2000).

Several ecological and epidemiological studies suggest that VRE could be transmitted from animals to humans through the food chain (Klein *et al.,* 1998; Woodford *et al.,* 1998; Lemcke and Buelte 2000; Robredo *et al.* 2000). The water cycle has been suggested as the transmission route for some resistance to antibiotics (Kühn *et al.* 2000; Iversen *et al.* 2002).

In this study, the enterococcal populations in various raw and treated urban wastewaters, surface waters receiving the treated wastewaters and raw hospital wastewaters were analysed with regard to species distribution. Samples were taken from three European regions with different climates (central Sweden, southeast United Kingdom and northeast Spain) in order to determine whether there is a global species distribution in wastewaters in different geographic regions. VRE and ERE were also studied to evaluate the persistence of these groups in the wastewaters and to compare countries that had recently used (the United Kingdom and Spain until 1997) and had not recently used (Sweden since 1986) avoparcin and tylosin as animal growth promoters. The phenotypic diversity and similarity of enterococci populations were also calculated for each kind of wastewater and for each country. *Enterococcus* spp. isolates associated with resistance to the antibiotics vancomycin and erythromycin were also analysed.

MATERIALS AND METHODS

Sampling and enumeration of enterococci populations

Raw and treated urban wastewater samples (RW and TW, respectively) were collected at a number of urban wastewater treatment plants in Spain, Sweden and the United Kingdom. A total of 11 wastewater treatment plants were sampled. Most of them have biological secondary treatment but two are based only on physical and chemical treatments. Surface waters receiving the treated effluents of these plants (SW) were also sampled. Wastewater from hospitals (HW) was also sampled in each country. The sampling sites in each of the five hospitals were selected avoiding any disinfection of the raw wastewaters. Samples were taken using sterile bottles, and kept at 4°C for less than 6 h until they were processed (Anonymous 1994).

The enumeration of enterococci was performed by membrane filtration through 0·45-μm pore-size membranes (Millipore) in triplicate. Membranes were pre-incubated on Brain Heart Infusion Agar (BHIA; Difco) at 37°C for 2 h. This pre-treatment allowed the recovery of stressed cells (Anonymous 1998). One of the membranes was then transferred onto the selective medium *Enterococcus* Agar (MEA; Difco), another onto MEA supplemented with 8 mg l^{-1} of erythromycin (MEAE8) and the last membrane from the triplicate onto MEA supplemented with 8 mg l^{-1} of vancomycin (MEAV8). The last two plates, MEAE8 and MEAV8, provided enterococci isolates resistant to erythromycin (ERE8) and to vancomycin (VRE8), respectively. All of them were incubated at 37°C for 48 h. The confirmation of enterococci colonies was performed by transferring membranes onto Bile Esculin Agar (Difco, Detroit, USA) and incubating at 45°C for 1 h. A black colouration for the enterococci colonies signified hydrolysis of aesculin (Figueras *et al.* 1998; Manero and Blanch 1999). VRE8 isolates were spread on MEA supplemented with 20 mg l^{-1} of vancomycin (MEAV20) to determine their resistance at this concentration (VRE20 isolates). Quality control of the vancomycin and erythromycin media was achieved by inoculating plates with *Enterococcus faecalis* ATCC 29212 (vancomycin sensitive), *Ent. faecalis* FS18 (vancomycin-resistant *VanA* and erythromycin resistant, from Environmental & Public Health Unit, University of Brighton, UK;

Kühn *et al*. 2000) and *Ent. gallinarum* SMI 18658 (erythromycin sensitive, SMI: Swedish Institute for Infectious Diseases Control). Additionally, vancomycin-enrichment was performed on all samples in order to detect even low bacterial counts of VRE20. Ten millilitres of the wastewater samples were mixed with an equal volume of 2 concentrated vancomycin-enrichment broth (m-*Enterococcus* broth supplemented with 8 mg l^{-1} of vancomycin). After incubation for 24 h at 37°C, 10 μl of the enrichment broth were streaked on MEAV8 and MEAV20 and incubated at 37°C for 48 h. Colonies growing on MEAV20 plates were also considered VRE20 isolates for further biochemical phenotyping.

Biochemical fingerprinting

Twenty four colonies were picked randomly for each sample from MEA plates (Bianchi and Bianchi 1982; Kühn *et al.* 1997). These colonies were regarded as representative for the enterococci populations in each sample. The selected *Enterococcus* isolates were typed using the PhP-RF system (PhPlate Microplates Techniques AB, Sweden) according to the manufacturer's instructions. The PhP-RF plates are 96-well microplates with 11 dehydrated reagents, which provide a high level of discrimination among enterococci. The biochemical fingerprinting procedure with this system has been described earlier (Kühn 1985). The inoculated microplates were incubated at 37°C, and the absorbance value (A_{620}) of each well was measured 16, 40 and 64 h after inoculation with a microplate reader (Kühn and Möllby 1993). The biochemical fingerprinting of each isolate was calculated as the average of the absorbance values for each well over all three readings (Kühn *et al.* 1991). The similarity between strains was calculated as a correlation coefficient. Isolates presenting correlation coefficients higher than 0·975 were assigned to the same biochemical type. The population similarity (S_p) coefficient was used to calculate the similarities between *Enterococcus* populations in the waters from different sites (Kühn *et al.* 1991). The unweighted-pair-group method using average linkages (UPGMA) was applied for clustering of correlations and S_p coefficients (Sneath and Sokal 1973). The diversity of the bacterial populations studied was calculated using Simpson's diversity index (D_i) (Hunter and Gaston 1988). The optical readings, calculation of correlation coefficients, diversity indexes, population similarity values and clustering studies were performed using the PhpWin software (PhPlate Microplates Techniques AB, Sweden).

Species identification

The biochemical phenotyping by the PhP-system and the clustering analysis of almost 20 000 enterococcal isolates performed in an international research project (Kühn *et al.* 2000) provided a pool of 178 representative isolates of the different Phene Plate System biochemical types for each *Enterococcus* spp. These isolates were selected as representative because they showed the highest minimum and highest mean similarity to all other isolates belonging to the same type (Kühn *et al.* 1991). The species identification of all these representative strains was performed by standard methods and the *Enterococcus* matrix is described elsewhere (Manero and Blanch 1999), using Bacterial Identifier software (Blackwell Science Publishers Ltd., Oxford, UK). Nineteen species were considered when identifying representative isolates, based on the previous procedure: *Ent. asini, Ent. avium, Ent. casseliflavus, Ent. cecorum, Ent. dispar, Ent. durans, Ent. faecalis, Ent. faecium, Ent. flavescens, Ent. gallinarum, Ent. hirae, Ent. malodoratus, Ent. mundtii, Ent. pseudoavium, Ent. raffinosus, Ent. saccharolyticus* and *Ent. sulfureus*. The type collection strains of these *Enterococcus* spp. were also biochemically phenotyped using the Phene Plate System in order to compare their biochemical profiles with those of the environmental strains. Finally, the environmental representative isolates and the type strains were grouped, and the biochemical types related to each species. These relationships were used to identify the high number of enterococci strains from environmental studies. Identification was carried out by correlation of the biochemical types between each environmental strain with the representative isolates and type strains. The identification threshold was established when the correlation index was higher than 0·9. *Ent. casseliflavus, Ent. flavescens* and *Ent. gallinarum* have been suggested to be a single species by other authors (Teixeira *et al.* 1997; Patel *et al.* 1998; Quednau *et al.* 1998; Baele *et al.* 2000). Because of this and the lack of consistent phenotypic differentiation between them, they were considered a unique group of species (CFG group) in this study.

RESULTS

Four hundred and six samples were taken: 105 and 102 from raw and treated urban wastewater, respectively, 140 from surface waters receiving treated wastewater and 59 from hospital wastewaters (Table 1). The levels of enterococci were around 10^6 CFU per 100 ml^{-1} in raw wastewater and usually 1·5–2·5 orders of magnitude lower in treated wastewater for any studied sample. A wider range of counts (10^1–10^4 CFU per 100 ml^{-1}) was found for the sampled surface waters. Hospital wastewater showed a similar enterococci count (10^5 CFU per 100 ml^{-1}) between countries.

A total of 9296 strains were subjected to biochemical fingerprinting using the Phene Plate System. The diversity index (D_i) was calculated by pooling all the isolates

Table 1 Total number of samples and phenotyped isolates from different samples in the three countries, and their enumeration and diversity index

	Number of samples	Counts	Number of phenotyped isolates	D_i	Percentage of prevalence of resistance		
					ERE8	VRE8	VRE20
Sweden							
RW	35	5·60 (0·35)	869	0·97	26	57	57
TW	31	2·89 (1·15)	677	0·96	65	19	19
SW	35	1·33 (0·63)	625	0·96	63	3	3
HW	14	5·95 (0·65)	373	0·93	86	43	36
Spain							
RW	49	6·01 (0·47)	1329	0·95	100	98	90
TW	50	4·38 (1·04)	1196	0·96	88	62	54
SW	67	2·57 (0·98)	1202	0·94	69	18	13
HW	23	5·39 (0·96)	488	0·95	83	30	22
UK							
RW	21	6·13 (0·46)	520	0·97	NA	67	52
TW	21	4·87 (0·67)	299	0·96	NA	29	29
SW	38	3·15 (0·89)	255	0·92	NA	21	11
HW	22	5·08 (1·26)	210	0·97	NA	18	5

RW, raw wastewater; TW, treated wastewater; SW, receiving surface water; HW, hospital wastewater. D_i index of diversity for the enterococci in each sample type. Counts are indicated as $\log_{10}$ of CFU per 100 ml^{-1}, and the standard deviation is indicated in brackets. The total number of phenotyped isolates coming from m-*Enterococcus* agar plates is indicated for each kind of sample. The prevalence of resistance is indicating by the percentage of samples that presented resistant isolates for the different antibiotics: ERE8, enterococci resistant to 8 mg l^{-1} of erythromycin; VRE8, enterococci resistant to 8 mg l^{-1} of vancomycin; VRE20, enterococci resistant to 20 mg l^{-1} of vancomycin.
NA, not analysed.

belonging to the same type of water and country of origin. D_i values were always equal to or higher than 0·92 (Table 1). Similar diversities were also normally obtained for ERE8, VRE8 and VRE20 isolates independently of the country of origin, although in some cases D_i was lower than 0·9 (0·84 for Swedish ERE8 isolates and 0·88 for Spanish VRE20 isolates). A high proportion of samples (independent of the country of origin) contained ERE8 isolates (Table 1). VRE strains were isolated in all three countries, but they showed a lower prevalence than the ERE8 isolates. The prevalence of ERE8 isolates in the analysed samples was very high for all the countries studied (Table 1). A lower percentage of samples contained VRE8 and VRE20 isolates but they were still present in all countries.

There was a high enterococcal population similarity ($S_p \geq 0{\cdot}25$) between the samples of the three countries when the different types of water were compared. The similarity between enterococcal populations was also analysed for each country (Fig. 1). High similarities were found among enterococcal populations from Spanish and Swedish wastewaters ($S_p \geq 0{\cdot}43$ and $S_p \geq 0{\cdot}34$, respectively). In all the countries, the highest similarity was found between raw and treated urban wastewater (Fig. 1). The hospital wastewater in all three countries showed the lowest similarity to other sample types. The comparison of ERE8 populations between countries also showed a high similarity ($S_p \geq 0{\cdot}37$) between Swedish and Spanish populations. No significant similarities were found when comparing VRE8 populations between countries, the S_p values being equal or lower than 0·09 (Fig. 2). However, VRE20 populations were similar in all countries, although a higher S_p index was found between the United Kingdom and Spanish isolates (Fig. 2). ERE8 populations were more similar between countries ($S_p > 0{\cdot}34$) than VRE8 or VRE20 populations ($S_p < 0{\cdot}09$ and $S_p < 0{\cdot}22$, respectively).

Most of the enterococci strains isolated in any type of water studied belonged to the species *Ent. faecalis* or *Ent. faecium* (Table 2) together usually representing more than 60% of the total enterococcal population. *Ent. hirae* was the next most prevalent followed by the CFG group and *Ent. durans*. *Enterococcus* spp. that were recorded at percentages lower than 2% in all countries and sample types were included in the group 'other' (Table 2). Swedish isolates presented an inverse percentage for *Ent. faecalis* and *Ent. faecium* with respect to Spanish and UK isolates.

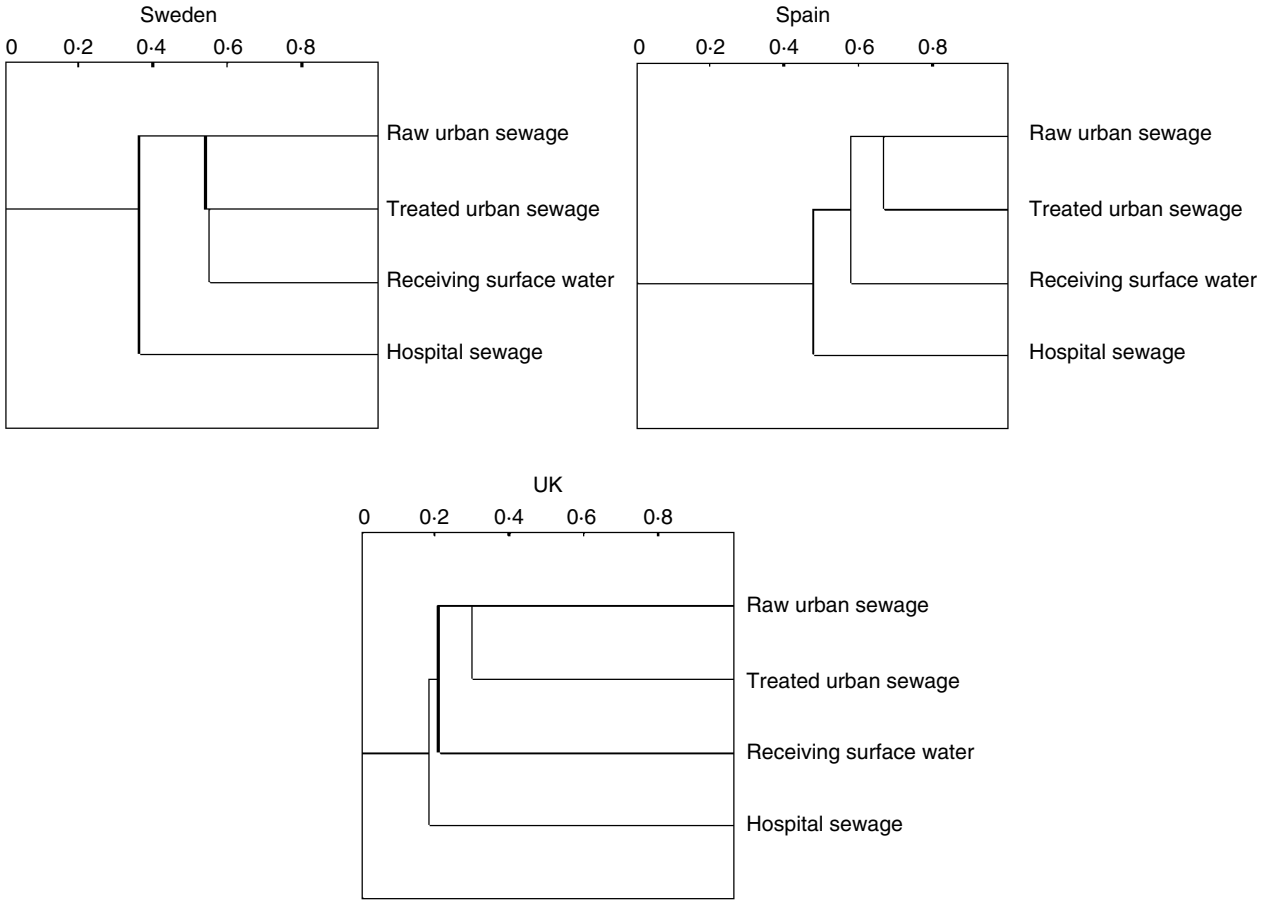

Fig. 1 Dendrograms of the clustering analysis of the similarity indices between the pooled isolates of each kind of wastewater studied for the different countries

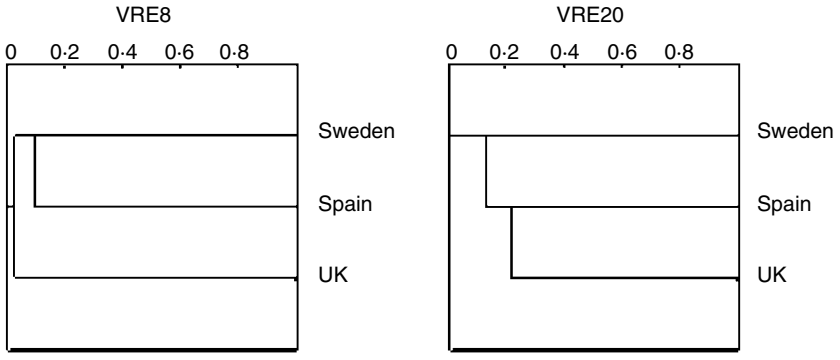

Fig. 2 Dendrograms of the clustering analysis of the similarity indices between the pooled VRE8 and VRE20 isolates of each country

Ent. faecalis, *Ent. faecium* and *Ent. hirae* were also the most common species among ERE8 in all countries (Table 2). *Ent. faecalis* was the most abundant ERE8 species in Sweden, and it was found at similar percentages to *Ent. faecium* among ERE8 Spanish isolates.

Ent. faecium was the most frequent species among VRE isolates in all countries. Other VRE species were *Ent. faecalis*, the CFG group and *Ent. hirae* but at lower percentages (Table 2). A higher percentage of *Ent. faecium* (70%) was found among VRE8 in the United Kingdom compared with the other countries (43 and 48% for Sweden and Spain, respectively). *Ent. faecium* consistently represented more than 60% of the VRE20 isolates in any country. However, Swedish isolates represented a higher percentage

Table 2 Percentages of the *Enterococcus* spp. for each type of sample and country

	RW			TW			SW			HW			Total		
	S	E	UK	S	E	UK	S	E	UK	S	E	UK	S	E	UK
ENT															
CFG	5	1	2	4	3	3	2	2	1	3	2	1	4	2	2
Ent. durans	2	1	0	2	1	0	3	1	7	0	0	0	2	1	1
Ent. faecalis	44	33	22	35	37	44	38	22	1	53	23	30	41	30	24
Ent. faecium	24	41	62	28	36	43	29	48	62	24	49	47	27	43	55
Ent. hirae	17	19	8	21	17	4	20	24	22	8	22	2	18	20	9
Other	8	4	7	9	7	5	8	4	7	11	5	21	8	5	9
ERE8															
CFG	0	1	NA	0	2	NA	0	1	NA	0	0	NA	0	1	NA
Ent. durans	0	1	NA	4	0	NA	2	1	NA	0	0	NA	2	0	NA
Ent. faecalis	76	52	NA	70	53	NA	69	41	NA	84	42	NA	75	48	NA
Ent. faecium	14	36	NA	18	39	NA	19	44	NA	10	52	NA	15	41	NA
Ent. hirae	8	7	NA	2	5	NA	5	11	NA	4	4	NA	5	7	NA
Other	2	4	NA	6	1	NA	5	3	NA	2	1	NA	4	3	NA
VRE8															
CFG	5	9	0	0	50	0	0	25	0	13	0	0	6	13	0
Ent. durans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ent. faecalis	32	0	3	0	0	0	100	0	0	0	25	20	21	10	6
Ent. faecium	41	36	76	46	25	0	0	75	0	50	58	40	43	48	70
Ent. hirae	3	0	0	39	0	0	0	0	0	0	0	0	9	0	0
Other	19	55	21	15	25	0	0	0	0	38	17	40	22	29	24
VRE20															
CFG	9	4	0	0	5	0	0	0	0	0	15	0	6	5	0
Ent. durans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ent. faecalis	26	7	7	33	1	16	0	0	0	0	0	20	23	4	10
Ent. faecium	61	85	90	33	89	68	100	80	70	100	85	70	63	86	78
Ent. hirae	0	1	0	33	0	0	0	10	0	0	0	10	6	1	1
Other	4	4	3	0	5	16	0	10	30	0	0	0	3	4	10

RW, raw wastewater; TW, treated wastewater; SW, receiving surface water; HW, hospital wastewater.
ENT, total enterococci isolates from m-*Enterococcus* agar; ERE8, enterococci resistant to 8 mg l^{-1} of erythromycin.;VRE8, enterococci resistant to 8 mg l^{-1} of vancomycin; VRE20, enterococci resistant to 20 mg l^{-1} of vancomycin; CFG, *Ent. casseliflavus, Ent. flavescens* and *Ent. gallinarum* group.
Other: *Enterococcus* spp. in a percentage lower than 2% for any country or any kind of sample or non-identified.
S, Sweden; E, Spain; UK, United Kingdom.
NA, not analysed.

for *Ent. faecalis* (almost 23%) in comparison with the Spanish and UK VRE20 isolates (4 and 10%, respectively).

DISCUSSION

Results from the wastewater treatment plants were similar in the countries studied, both with regard to the total counts of enterococci and with regard to the reduction capacity of the numbers of enterococci during the treatment process. A higher variability between countries was detected for the numbers of enterococci in surface waters. The wider range of values in these waters could be explained because different receiving surface waters were sampled. The hospital wastewaters also showed similar values.

All studied wastewaters also contained diverse enterococcal populations, and showed rather similar structure and composition when comparing enterococcal populations in different wastewaters. The types of treatment systems used in plants at the different regions did not appear to affect results. Hospital wastewater in all three countries showed lower similarities to the urban wastewaters and receiving surface waters. This could be explained by the fact that, the bacteria in hospital wastewater are derived from a particular fraction of the human population, and that the hospital environment is selective for certain bacterial strains. This fact is supported by similar results in each country studied.

The chosen procedure for the identification of species, by comparison of the PhP-system results to a reference

database of PhP-typing data from strains of known species and by choosing a level of similarity higher than 0·9 for positive identification, provided a successful method for the determination of species. Less than 7% of the studied isolates from MEA were not identified using this approach. Seven isolates that were classified as *Ent. faecium* according to their PhP typing data should be classified as *Ent. mundtii*. However, they could easily be species differentiated since they produced bright yellow colonies.

Ent. faecalis and *Ent. faecium* are generally the most common enterococcal species in the animal and human intestinal flora (Devriese *et al.* 1987, 1992) and thus also the most common species in hospital and urban wastewaters (Pourcher *et al.* 1991; Laukova and Juris 1997; Svec and Sedlacek 1999; Manero *et al.* 2002). In this study, they were also the most common species in the samples of all three countries, and in all samples types. However, *Ent. faecium* was more abundant than *Ent. faecalis* in the UK and Spanish samples, but the proportion was inverted in all sample types from Sweden. This latter observation is more in line with the findings of other studies (Pourcher *et al.* 1991; Valdivia *et al.* 1996), which also described a higher prevalence of *Ent. faaecalis*. Other common enterococcal species were *Ent. hirae,* the CFG group and *Ent. durans*. *Ent. cecorum* was not detected in any sample because of the requirement for incubation in an atmosphere containing 5% CO_2.

Very little is known about regional differences in the composition of the bacterial flora in individuals and in wastewater. Most previous studies on the topic have dealt with observations from a unique country or region. It is possible that differences in climate or diet regimes between the studied countries could contribute to the differences in the predominance of certain *Enterococcus* spp. This could justify the observed differences between Sweden and the other countries.

The screening for VRE (or other antibiotic resistance) in individual faecal samples is a cumbersome procedure, especially when the prevalence of VRE is low, but since wastewaters consist of mixtures of faecal flora from many individuals, isolates with certain properties may be found more easily. The enterococcal population structure of the studied wastewater also seemed to resemble closely that of a large population of individual samples. An important finding was the quite high prevalence of VRE in Swedish urban and hospital wastewater. This would have remained undiscovered without the sampling approach performed in this study. *Ent. faecium* was the most common species among VRE8 isolates followed by *Ent. faecalis* and the CFG group in all countries. Some *Ent. hirae* VRE8 strains were isolated in Sweden but were never isolated in the other countries. Comparisons of the VRE8 isolates from the three countries indicated that no specific types were prevalent, and thus different clones seem to exist in the different geographic areas. Also, a high diversity was observed for the VRE8 populations within each of the three countries. Most of the VRE8 isolates were confirmed as VRE20 isolates. Such strains were isolated in all the studied countries. All samples in the present study were collected during the years 1998–2000. The time elapsed since glycopeptide usage in animal production was banned at the sites studied (since 1986 in the Swedish region; 1996 in the Spanish region and 1997 in the British region) does not support the prevalence of VRE20 in the environment, particularly in Sweden, and in wastewater as argued by other authors (Borgen *et al.* 2000). Another explanation could be that clinical overuse of antimicrobial glycopeptides or co-selection by the continued use of other growth promoters in animal production have caused the high prevalence of VRE20 in wastewater (Bates 1997; Aarestrup 2000). It is also important to remember that vancomycin is still used for treatment of human infection. Additionally, it was observed that most of the VRE20 isolates showed also erythromycin resistance that could favour the persistence of vancomycin resistance in the environment. The resistance to the macrolide erythromycin was common in all the investigated areas. As this antibiotic is widely used in both animal production and human medicine, one may conclude that the selective pressure from both environments could cause the high prevalence of resistant strains.

In conclusion, the sampling of urban and hospital wastewaters provided a feasible way to analyse the distribution of *Enterococcus* spp. in different areas, as well as the prevalence of antibiotic resistant strains. These kinds of samples could be a practical target for surveillance of the prevalence of ERE and VRE isolates in the environment. This study shows these bacteria can pass through different types of treatment in wastewater plants and consequently they could be transferred to surface waters increasing the chance of being transmitted via the food chain.

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DISCUSSIÓ.

La proporció observada en les 5 estacions depuradores d'aigües residuals (EDARs) entre coliforms fecals, enterococs totals, els resistents a l'eritromicina (ERE) i els resistents a la vancomicina (VRE) va ser molt semblant a les aigües residuals abans i després de tractar, independentment de l'origen de l'aigua residual, el tipus de tractament o l'eficiència de reducció. No obstant, es van detectar menors reduccions d'espores de clostridis respecte coliforms fecals i enterococs en les aigües tractades d'aquelles EDARs amb temps de retenció hídrica més llargs (plantes 1, 2 i 3). Pel que fa a les plantes amb un temps de retenció hídrica curt (plantes 4 i 5) la reducció va ser molt semblant per tots els indicadors analitzats. Aquests resultats pel que fa a coliforms fecals, enterococs totals i espores de clostridis sulfit-reductors confirmen les observacions fetes en altres estudis a diferents països (**Hill i Sobsey 1998; Lucena i col. 2004**; **Payment i Franco 1993**). La major persistència de les espores de clostridis sulfit-reductors a l'ambient descrita per diversos autors (**Scheuerman i col. 1987; Barcina i col. 1990; Sinton i col. 1994; Mocé-Llivina i col. 2003; Tree i col. 2003**) podria explicar aquest fenomen, però també és possible que els majors intervals d'airejament en les EDARs amb un temps de retenció llarg indueixin a l'esporulació dels clostridis, mentre que en les EDARs amb temps de retenció curt, aquest no sigui suficient per produir aquesta inducció (**Lucena i col. 2004**).

Les resistències a l'eritromicina i a la vancomicina es van detectar en unes proporcions del 10 % i el 0,05% dels enterococs totals respectivament. Aquestes proporcions es van observar en tots els tipus de mostres analitzades, independentment de l'origen, el tractament o la càrrega microbiana. Aquests resultats recolzen la gran similitud entre les aigües residuals abans i després dels diferents tractaments i suggereixen que no hi ha cap factor, ni en les àrees d'influència de les depuradores ni en els processos de tractament, que produeixin una pressió de selecció sobre els enterococs resistents als antibiòtics estudiats. Aquests resultats contrasten amb l'increment detectat en la proporció de coliforms fecals resistents a antibiòtics a l'aigua tractada descrit en altres estudis (**Mezrioui i Baleux 1994; Andersen 1993**).

Les poblacions microbianes estudiades en totes les aigües presentaven una elevada diversitat. Aquesta sempre fou superior en els coliforms fecals que en els enterococs. La similitud poblacional fou també alta en ambdós grups microbians, però

superior en els enterococs que en els coliforms fecals. Aquesta circumstància pot explicar-se perquè la definició del terme coliforms fecals inclou diversos gèneres i espècies de bacteris, mentre que en el cas dels enterococs es tracta d'espècies d'un sol gènere (*Enterococcus*).

Els alts valors de similitud entre les aigües residuals abans i després de passar per les EDARs, demostren també que no hi ha una eliminació selectiva en les poblacions dels indicadors estudiats al llarg dels processos de tractament. Les espècies predominants en els coliforms fecals van ser, *E. coli* i *Citrobacter* spp. La primera representa entre el 50 i el 75% dels perfils bioquímics identificats i entre el 14 i el 29% del total de les soques fenotipades. *Citrobacter* spp. representa entre el 20 i el 50% del perfils bioquímics identificats i entre el 7 i el 18% del total dels perfils bioquímics. Aquestes dades concorden amb d'altres estudis realitzats (**Brown i Tracey 1975; Mersch-Sunderman i Wundt 1987; Hill i Sobsey 1998: McLellan i col. 2001**). En el cas d'enterococs, *Ent. faecium* i *Ent. faecalis* van ser les espècies predominants en totes les mostres analitzades. Aquestes observacions concorden amb els treballs de **Laukova i Juris 1997** i de **Svec i Sedlacek 1999**. També són semblants als obtinguts per **Sinton i Donnison** (**1994**) a l'estudiar la composició dels enterococs en diferents aigües residuals de Nova Zelanda on *Ent. faecium* i *Ent. faecalis* eren també, per aquest ordre, les espècies dominants a les aigües residuals d'origen humà analitzades per aquests darrers autors. Així mateix, la proporció d'*Ent. faecium* fou lleugerament superior a la d'*Ent. faecalis* en la majoria de mostres analitzades, excepte a l'entrada de la planta 5, a la planta 4, i especialment a l'entrada de la planta 1. Cal destacar que l'origen de les aigües residuals d'aquesta darrera planta era mixt, amb importants aportacions d'indústries agroalimentàries, en una zona on el sector porcí té una gran activitat. Aquest fet podria explicar perquè en les mostres d'entrada d'aquesta depuradora, la proporció d'ambdues espècies és semblant a les observades en les poblacions d'enterococs de purins, descrites per **Manero i col. (2002).**

Els perfils bioquímics de les poblacions d'enterococs resistents a l'eritromicina van ser classificats majoritàriament com a *Ent. faecium* i *Ent. faecalis*. Els perfils bioquímics classificats com a *Ent. faecalis* foren més abundants que els d'*Ent. faecium*, tal i com passava a les mostres de l'entrada de la depuradora 1, i les de purins descrites

per **Manero i col.** (**2002**) Aquest fet podria assenyalar les explotacions ramaderes com a possible origen d'aquesta resistència d'acord amb altres autors (**Jackson i col. 2004**). Contràriament, els perfils bioquímics de les poblacions d'enterococs resistents a la vancomicina van ser classificats majoritàriament com a *Ent. faecium*. A més a més, els perfils bioquímics de les soques pertanyents a *Ent. gallinarum*, degut a la seva resistència intrínseca a la vancomicina (**Bridge i Sneath, 1982**), foren el segon grup en percentatge. Tot i això, les proporcions d'espècies associades als perfils bioquímics majoritaris de les poblacions d'enterococs resistents a la vancomicina són diferents a les de la resta de poblacions d'enterococs.

Les proporcions i les concentracions dels tres indicadors, inclús la dels enterococs resistents a l'eritromicina, foren molt semblants a l'aigua tractada de la depuradora 3, riu amunt i els dos punts riu avall del punt d'emissió. Aquests resultats contrasten amb d'altres estudis ja esmentats en la introducció pel que fa a la diversitat de les poblacions microbianes de les mostres analitzades (**Kühn i col. 1997**). Pel que fa a la inactivació dels diferents indicadors microbians en aigües de riu altres autors descriuen diferències significatives entre el grau d'inactivació de coliforms, enterococs i clostridis (**Medema i col. 1997**). Tot i tractar-se d'un riu mediterrani sembla que amb les dades dels cabals (veure pàgina 19) caldria esperar una de dilució de la càrrega microbiana, però les aigües riu amunt ja presenten una càrrega semblant a la de l'emissari. És possible que la càrrega sigui massa elevada perquè els mecanismes d'autodepuració puguin reduir-la substancialment (**Curds 1992**) o potser la distància entre els punts analitzats no sigui suficient per detectar aquesta reducció. Per la mateixa raó es podria explicar que el comportament dels diferents indicadors no presenti diferències significatives al llarg dels diferents punts del riu analitzats, al contrari del que s'esperaria tenint en compte els altres estudis en que els enterococs presenten una persistència intermèdia entre els coliforms fecals i les espores de clostridis sulfit-reductors (**Mocé-Llivina i col. 2003; Tree i col. 2003; Scheuerman i col. 1987; Durán i col. 2002**).

La baixa càrrega d'enterococs resistents a la vancomicina, sumada a la menor regularitat del cabal del riu respecte a l'emissari de la depuradora, fan difícil la comparació d'aquestes poblacions entre els dos grups de mostres. No obstant, cal

assenyalar que la proporció d'enterococs resistents a l'eritromicina va estar sempre al voltant del 10% del total en totes les mostres i que tant a la sortida de la depuradora com en mostres agafades riu avall de l'emissari es va detectar la presència d'enterococs resistents a la vancomicina.

En les mostres de riu i les de la depuradora 3 hi ha una elevada similitud entre tongades de mostres pel que fa a l'estructura i la composició de les dues poblacions bacterianes estudiades. No obstant, es defineixen 2 grups de mostres en les anàlisis d'agrupació. L'existència de dos fluxos d'aigua (el procés de tractament i el corrent del riu) podrien explicar aquests resultats. Al contrari del que passava en els estudis de **Kühn i col.** (**1997**) on valoren els abocaments d'aigües residuals industrials de papereres en un riu, els abocaments de l'emissari estudiat en el riu Ter no modificaren la composició de les poblacions de coliforms fecals ni les d'enterococs perquè els dos fluxos d'aigua compartien unes poblacions bacterianes similars. Aquestes hipòtesis estan recolzades per la comparació dues a dues de les soques agrupades per punts, que indicaren una elevada similitud dels 5 punts, tot i que s'agrupaven les mostres de riu per un costat i les de la depuradora per un altre (Figura 1 del capítol 2 de Resultats). Aquesta elevada similitud entre les 5 mostres podria ser deguda a l'existència d'un altre efluent riu amunt, presumiblement d'aigües residuals tractades.

Durant el període estudiat, l'emplaçament de la planta depuradora no afectà la càrrega, l'estructura ni la composició de les poblacions de coliforms fecals i d'enterococs del riu. Ambdues poblacions presentaren una distribució homogènia en les aigües estudiades, i en conseqüència, no sembla que estiguessin actuant sobre aquestes poblacions, factors de selecció pel seu creixement o eliminació.

Mentre que els coliforms fecals presenten les concentracions més elevades en aigües residuals, abans i després del tractament, seguida dels enterococs, els tres indicadors estudiats es trobaren en concentracions similars en els fangs. Per tant, en el fang els coliforms són eliminats amb una proporció més elevada que els enterococs i encara més que les espores de clostridis sulfit-reductors. Aquesta observació està en

concordança amb estudis previs fets per altres autors (**Fujioka i col. 1981; Scheuerman i col. 1987; Durán i col. 2002; Mocé-Llivina i col. 2003; Tree i col. 2003**) segons els quals la inactivació de coliforms fecals és major que la d'enterococs i alhora aquesta és major que la d'espores de clostridis sulfit-reductors. Ara bé, les semblances entre la composició i l'estructura de les poblacions microbianes estudiades a l'aigua residual abans i després del tractament, així com les diferències entre la composició i l'estructura d'aquestes poblacions i les dels fangs, suggereixen que sobre els indicadors microbians atrapats als fangs actuen uns mecanismes d'inactivació diferents, o si més no, que ho fan en un grau diferent que sobre les poblacions que queden a l'aigua.

Un dels principals factors proposats com a responsable de la reducció de la càrrega orgànica en el procés de tractament, és l'adsorció del material soluble i els microorganismes als flocs, els quals al sedimentar retiren del sistema una bona part de la càrrega orgànica inicial (**Curds 1992**; **Bitton 1994**). Dins els fangs, un dels principals factors de la reducció de la càrrega orgànica i microbiana és la predació i la síntesi de nova biomassa cel·lular (**Curds 1992**; **Bitton 1994**). Segons les observacions fetes per **González i col.** (**1990**), aquesta predació afecta més als gram negatius (coliforms fecals) que als gram positius (enterococs). Per tant, els resultats obtinguts estarien d'acord amb aquestes tres premisses. Així doncs, en un primer estadi, les poblacions microbianes quedarien "atrapades" en els fangs i un cop allà altres factors, com ara la predació per part dels protozous, propiciarien els canvis en les proporcions i l'estructura d'aquestes poblacions.

El lleuger increment de la proporció d'espores de clostridis sulfit-reductors a l'aigua tractada podria ser degut a la inducció de l'esporulació durant el procés d'airejament. Recolza aquesta hipòtesi el fet que la càrrega d'espores de clostridis sulfit-reductors al fang de recirculardització, fos superior a la de l'aigua d'entrada, cosa que no passava ni amb els coliforms fecals ni amb els enterococs. Sembla poc probable que aquest fet es doni per una major eliminació de coliforms fecals i enterococs a l'aigua, ja que tant les similituds poblacionals com les proporcions d'aquests dos indicadors es mantenen idèntiques a l'aigua de sortida, no essent així en els fangs. A més la concentració d'espores de clostridis sulfit-reductors als fangs de recirculardització és de $1,2 \times 10^6$ UFC/100ml, superior al resultat de multiplicar la concentració que presenten a

l'aigua residual que entra a la depuradora (3,7 x 10^5 UFC/100ml) per l'increment (2,5 vegades més de mitjana) del grau d'hidratació dels fangs de recirculardització respecte l'aigua d'entrada. Caldria considerar encara que la metodologia utilitzada pel recompte en fangs pot detectar un percentatge menor del real que no pas a l'aigua, degut als fenòmens d'adsorció de les cèl·lules i tenint en compte que seria esperable un cert grau d'inactivació.

En canvi, les concentracions microbianes dels 3 indicadors dels fangs de recirculardització i dels fangs premsats guarden entre ells una proporcionalitat amb el percentatge de sequedat, corresponent a un increment d'aproximadament 1,5 logaritmes del pes sec dels fangs (veure pàgina 19) amb aproximadament 1,5 logaritmes d'increment en la concentració dels tres indicadors (Taula 1 del capítol 3 de Resultats).

La proporció d'enterococs resistents a l'eritromicina en els fangs és molt semblant a la detectada a l'aigua residual de les altres EDARs. Tot i que els enterococs resistents a vancomicina es van trobar en baixes proporcions, aquests persisteixen després del procés de tractament de les aigües residuals i especialment en els fangs, cosa que s'hauria de tenir en compte tant en la reutilització de l'aigua tractada com en el destí dels fangs.

Pel que fa a les poblacions d'enterococos, ERE i VRE analitzades en aigües residuals urbanes i d'hospital de diferents regions europees es van detectar similituds importants. En primer lloc, a nivell quantitatiu, cal dir que la presència d'ERE a les mostres d'aigües residuals d'Espanya, Suècia i el Regne Unit va ser alta, mentre que es va detectar una baixa presència de VRE. No obstant, aquests enterococs resistents a la vancomicina es van detectar a tots 3 països. Les poblacions d'enterococs i ERE de Suècia i el Regne Unit presentaren una elevada similitud poblacional amb les espanyoles, essent màxima entre les sueques i les espanyoles. Pel contrari, la similitud entre les poblacions de VRE fou baixa, especialment pel que fa a les soques resistents a 20 mg l^{-1}de vancomincina. No obstant, la màxima similitud fou en aquest cas entre les poblacions espanyoles i angleses.

Les proporcions d'espècies d'enterococs predominants a les mostres de Suècia i Regne Unit foren també *Ent. faecium* i *Ent. faecalis*, a excepció d'algunes mostres d'aigües de riu on *Ent. hirae* fou més abundant que *Ent. faecalis*, però menys que *Ent. faecium*. En qualsevol cas entre *Ent. faecium* i *Ent. faecalis* sempre representaren més del 50% del totals de soques de les diferents mostres. Aquestes observacions concorden també amb d'altres estudis realitzats (**Laukova i Juris 1997; Svec i Sedlacek 1999**; **Sinton i Donnison 1994**). Pel que fa als ERE, *Ent. faecalis* va ser l'espècie dominant en totes les mostres dels tres països, seguida per *Ent. faecium*. En canvi, *Ent. faecium* va ser l'espècie dominant en les poblacions de VRE 20 de les mostres dels tres països, i en la majoria de les mostres en el cas de les poblacions de VRE 8. Aquests resultats podrien indicar un origen animal en el cas de la resistència a l'eritromicina ja que en estudis realitzats en purins de porc la espècie d'enterococ predominant fou *Ent. faecalis* (**Manero i col. 2002**) i es detectà una major abundància d'ERE.

CONCLUSIONS.

- (1) Les aigües residuals (crues i tractades) procedents d'EDARs de diferents àrees geogràfiques presentaren uns elevats índex de diversitat i de similitud poblacional tant pels coliforms fecals com pels enterococs.

- (2) Un major temps de retenció de les aigües residuals en les plantes de fangs activats es relaciona amb el canvi de les proporcions dels coliforms fecals, enterococos i espores de clostridis sulfit-reductors, incrementant la proporció d'aquests darrers.

- (3) No es detectaren canvis en la composició de les poblacions d'enterococs resistents a la vancomicina i els enterococs resistents a l'eritromicina entre les diferents plantes, tal i com manifesta l'índex de similitud poblacional. Tampoc existeixen diferències significatives entre aquestes poblacions quan es comparen les aigües residuals crues i les tractades.

- (4) Aproximadament 1 de cada 10 enterococs és resistent a l'eritromicina, i 1 de cada 5.000 ho és a la vancomicina. Aquestes proporcions es mantenen en totes les mostres d'aigües residuals i llots. Per tant, aquests enterococs resistents no són eliminats completament pels processos de tractament.

- (5) Les aigües residuals tractades i les aigües receptores del riu estudiat tenen una composició semblant. L'impacte de les emissions de l'aigua tractada no provoca canvis significatius en les poblacions de coliforms fecals i enterococs de les aigües del riu estudiat.

- (6) No es detecta una disminució significativa de la càrrega microbiana a nivell dels indicadors analitzats en els 2 punts riu avall de l'emissari. Ni el factor dilució ni els processos d'autodepuració del riu no són suficients, amb la distància entre els punts analitzats.

- (7) Els fangs de depuradora presenten unes proporcions diferents dels indicadors estudiats a les mostres d'aigua possiblement degut a les diferents persistències dels indicadors.

- (8) Els coliforms fecals són la població menys persistent dins els fangs, seguits pels enterococs.

- (9) Tot i que els índexs de diversitat per les poblacions de coliforms fecals i d'enterococs són elevats, i que els índex de similitud poblacional són també alts entre totes les mostres d'aigua residual crua i tractada, fangs i aigües de riu analitzades, la similitud entre els fangs de depuradora i les mostres d'aigua residual de la depuradora amb aportacions d'indústries agroalimentàries respecte a la resta de mostres és menor pel que fa als dos grups bacterians estudiats.

- (10) Les espècies d'enterococs dominants en les aigües residuals, al riu i als fangs estudiats són *Ent. faecium* i *Ent. faecalis*, mentre que *E. coli* i *Citrobacter spp.* predominen entre els coliforms fecals, amb l'única excepció dels fangs deshidratats, on es van detectar baixes proporcions de *Citrobacter spp*. Els enterococs resistents a la vancomicina es van classificar majoritàriament com a *Ent. faecium*, mentre que en els resistents a l'eritromicina predominava *Ent. faecalis*.

- (11) Les poblacions d'enterococs de les mostres d'aigua residual urbana i d'hospital analitzades a Suècia, Regne Unit i Espanya presentaren una elevada similitud poblacional i hi predominaren les espècies *Ent. faecium* i *Ent. faecalis*.

- (12) Les soques d'ERE més abundants en l'estudi internacional foren classificades com a *Ent. faecalis*, mentre que en els cas de les VRE foren *Ent. faecium*.

- (13) Es van detectar soques resistents a l'eritromicina i a la vancomicina en tots tres països, essent sempre més abundants les soques d'ERE que les de VRE. Aquestes es detectaren en baixes proporcions però en tots els tipus de mostres analitzades.

- (14) S'ha observat la presència dels mateixos clons poblacionals d'ERE a 8mg/L d'eritromicina i VRE a 20 mg/L de vancomicina a les aigües residuals estudiades en tots tres països, aspecte que recolza un origen semblant d'aquestes resistències.

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ANNEX I

ALTRES TREBALLS I COL·LABORACIONS.

A més a més del compendi d'articles que composen aquesta Tesi doctoral, el treball presentat en aquesta memòria ha permès també la publicació dels següents articles i comunicacions:

Col·laboracions en d'altres articles:

- Manero, A.; Vilanova, X.; Cerdà-Cuéllar, M. and Blanch, A.R. (2002). Caracterization of sewage waters by biochemical fingerprinting of Enterococci. *Water Res*. **11**, 2831-2835.

- Kühn, I.; Iversen, A.; Burman, L.G.; Olsson-Liljequist, B.; Franklin, A.; Finn, M.; Aarestrup, F.; Seyfarth, A.M.; Blanch, A.R.; Vilanova, X.; Taylor, H.; Caplin, J.; Moreno, M.; Dominguez, L.; Herrero, I. A. and Möllby, R. (2003). Comparison of enterococcal populations in animals, humans, and the environment- a European study. *Enterococci in Foods. Funtional and Saftey Aspects* 133-145.

Comunicacions :

- Vilanova, X.; Manero, A.; Jofre, J. y Blanch, A.R. (1999). Diversidad de las poblaciones enterococales en estaciones depuradoras de aguas residuales. *XVII Congreso de la Sociedad Española de Microbiología*. Granada (pòster).

- Vilanova, X.; Manero, A.; Cerdà-Cuéllar, M.; Jofre, J. y Blanch, A.R. (2000). Efecto del emisario de una estación depuradora de aguas residuales en las poblaciones de enterococos y coliforms fecales de las aguas del río receptor. *III Reunió Científica de Microbiología del Medio Acuático*. Santiago de Compostela (comunicació oral).

- Vilanova, X.; Manero, A.; Cerdà-Cuéllar, M.; Jofre, J. y Blanch, A.R. (2000). The effect of a sewage treatement plant effluent on the faecal coliform and

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- Vilanova, X.; Manero, A. y Blanch, A.R. (2001). Diversidad de las poblaciones de coliforms fecales y enterococos en las aguas residuales y fangos de una estación depuradora. *XVIII Congreso de la Sociedad Española de Microbiología*. Alacant (pòster).

- Manero, A.; Vilanova, X.; Cerdà-Cuéllar, M; Jofre, J. y Blanch, A.R. (2000). Caracterización de las aguas residuales mediante fenotipado bioquímico de enterococos. *III Reunión Científica de Microbiología del Medio Acuático*. Santiago de Compostela (comunicació oral).

- Manero, A.; Vilanova, X.; Cerdà-Cuéllar, M; Jofre, J. y Blanch, A.R. (2000). Diferentiation of sewage waters by biochemical fingerprinting of enterococci. *Healt-Related Water Microbiology Symposium. International Water Asociation*. París (pòster).