UNIVERSITAT DE BARCELONA

Diversitat de comunitats heteròtrofes associades a les aigües de consum

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Memòria presentada per Laura Sala i Comorera per optar al grau de doctora per la Universitat de Barcelona

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Tesi realitzada sota la direcció del Dr. Anicet R. Blanch Gisbert i la Dra. Cristina García Aljaro al departament de Genètica, Microbiologia i Estadística de la Facultat de Biologia de la Universitat de Barcelona.

El director	La directora	La doctoranda
Dr. Anicet R. Blanch Gisbert	Dra. Cristina García Aljaro	Laura Sala Comorera

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A la meva família

i a tots vosaltres

que he conegut durant aquests anys,

que seran sens dubte inoblidables.

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Moment per començar a escriure els agraïments. Tenia planejat escriure aquestes línies en un lloc idíl·lic i relaxat: a la piscina que s'havia de construir, Sitges, Eivissa... que havíem d'anar-hi tots junts, aquí la raó de la meva insistència per anar-hi. Ara ja sabeu el secret, companys. En fi, al terrat de casa, també és un bon lloc per escriure aquestes línies.

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aunque para mí sea un mareo,
en verso te lo escribo
para que sea bien exclusivo.
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- Ja els hi donat prou menjar als pollets o bitxets (=bacteris) perquè puguin passar tot el cap de setmana?

- Avui els has congelat o els hi has donat menjar per passar el cap de setmana?

I per acabar a la meva família, a les correccions angleses de la Família Evans-Allué, als tiets, a la iaia, a la padrina i l'avi, als meus pares i a la Tresa.

ÍNDEX

LLISTAT D'ABREVIATURES

ACEA	Associació Catalana d'Envasadors d'Aigua
ADN	Àcid desoxiribonucleic
ANEABE	Associació Nacional d'Empreses d'Aigües de Beguda Envasada
AOC	Carboni orgànic assimilable
APPCC	Anàlisi de perills i punts de control crítics
ARNr	Àcid ribonucleic ribosòmic
C	Carboni
C	Citosina
Ca	Calci
CAG	Carbó actiu granular
$CaCO_3$	Carbonat càlcic
cm^2	Centímetres quadrats
CR	Clor residual lliure
CT	Clor total
Di	Diversitat de Simpson
et al.	i col·laboradors
FA	Àcid ferúlic
h	Hora
hm^3	Hectòmetres cúbics
HPC	Recompte d'heteròtrofs en placa
Da	Daltons
DGGE	Electroforesi amb gel de gradient desnaturalitzant
DHAP	2,6- dihydroxyacetophenone
DHB	Àcid 2,5-dihydroxybenzoic
DOC	Carboni orgànic dissolt
EFBW	Federació europea d'aigües envasades
EPA	Agència de protecció ambiental d'Estats Units
ETAP	Estació de tractament d'aigua potable

G	Guanina
GSP	Medi glutamat midó de fenol
HCCA	Àcid alfa-ciano-4-hidroxicinamic
l	Litre
ISO	Organització Internacional d'Estandardització
MALDI-TOF MS	Espectrometria de masses de desorció/ionització mitjançant làser assistida per matriu acoblada a un analitzador de temps de vol
m^3	Metres cúbics
m/z	Relació massa/càrrega
min	Minut
Mg	Magnesi
mg	Mil·ligram
ml	Mil·lilitre
MLSA	Anàlisis multilocus de les seqüències
N	Nitrogen
nm	Nanòmetre
O	Oxigen
OI	Osmosi inversa
p	Variable de significació estadística
P	Fòsfor
PCA	Anàlisi de components principals
pb	Parells de bases
PCR	Reacció en cadena de la polimerasa
PFGE	Electroforesi en gel de camp polsat
pH	Potencial d'hidrogen
PET	Tereftalat de polietilè
PVC	Clorur de polivinil
r	Coeficient de correlació
RAPD	Amplificació aleatòria d'ADN polimòrfic
s	Segon
SA	Àcid sinapínic

SEM	Microscopi electrònic de rastreig
Sp	Coeficient de similitud poblacional
sp.	Espècie no determinada
spp.	Diverses espècies
T	Temperatura
UFC	Unitat formadora de colònia
UNF	Unitats nefelomètriques de terbolesa
UPGMA	en anglès, *Unweighted Pair Group Method with Arithmetic mean*
V	Volts
µm	Micròmetre
YEA	Extracte de llevat agar
˚C	Graus Celsius
€	Euro
%ID	Percentatge de la precisió d'identificació

1.INTRODUCCIÓ

1.INTRODUCCIÓ

L'aigua és una molècula essencial per a la vida, tot ésser viu necessita un requeriment mínim d'aigua. Sens dubte, l'aigua ha estat un element que ha marcat el desenvolupament de totes les civilitzacions i concretament la cerca d'aigua saludable per a la ingestió de les persones, sigui provinent de pous, rius, fonts... i és que l'aigua és un vincle de transmissió de moltes malalties. De fet, les grans epidèmies i la principal causa de mortalitat, fins a principis del segle XX ha estat deguda a les malalties infeccioses de transmissió hídrica: còlera, shigel·losi, febre tifoide... (Jofre, 2010).

El desenvolupament i la millora de les tècniques de tractament i de potabilització de l'aigua han estat fonamentals per a la disminució de les malalties de transmissió hídrica i per a la millora de la qualitat sanitària de la població. En aquest aspecte, el control de la qualitat microbiològica de l'aigua a partir dels indicadors de la contaminació fecal ha contribuït especialment a detectar el risc associat a la possible presència de patògens degut a una contaminació fecal.

Deixant de banda els microorganismes patògens que pot contenir l'aigua i que han estat l'objecte de molts estudis, l'aigua conté una comunitat microbiana que no comporta, en principi, cap risc per a la salut, doncs els medis aquàtics d'aigua dolça alberguen la major diversitat bacteriana entre els diferents hàbitats naturals. Així és com en l'aigua subterrània, els aqüífers, els rius, els llacs i l'aigua residual trobem el nombre més gran i més divers de llinatges bacterians (Tamames et al., 2010). Els fílums més abundants, en ordre decreixent, són *Proteobacteria, Actinobacteria, Bacteroidetes* i *Firmicutes,* independentment del tipus d'aigua (Fig. 1a, 1b).

La diversitat pròpia de l'aigua de consum ha estat poc explorada fins el moment. El propòsit d'aquesta tesi doctoral és contribuir a l'estudi de la composició i la dinàmica de les comunitats microbianes de diferents tipus d'aigua de consum humà: aigua mineral natural envasada i aigua de xarxa de distribució al llarg del procés de potabilització.

1a

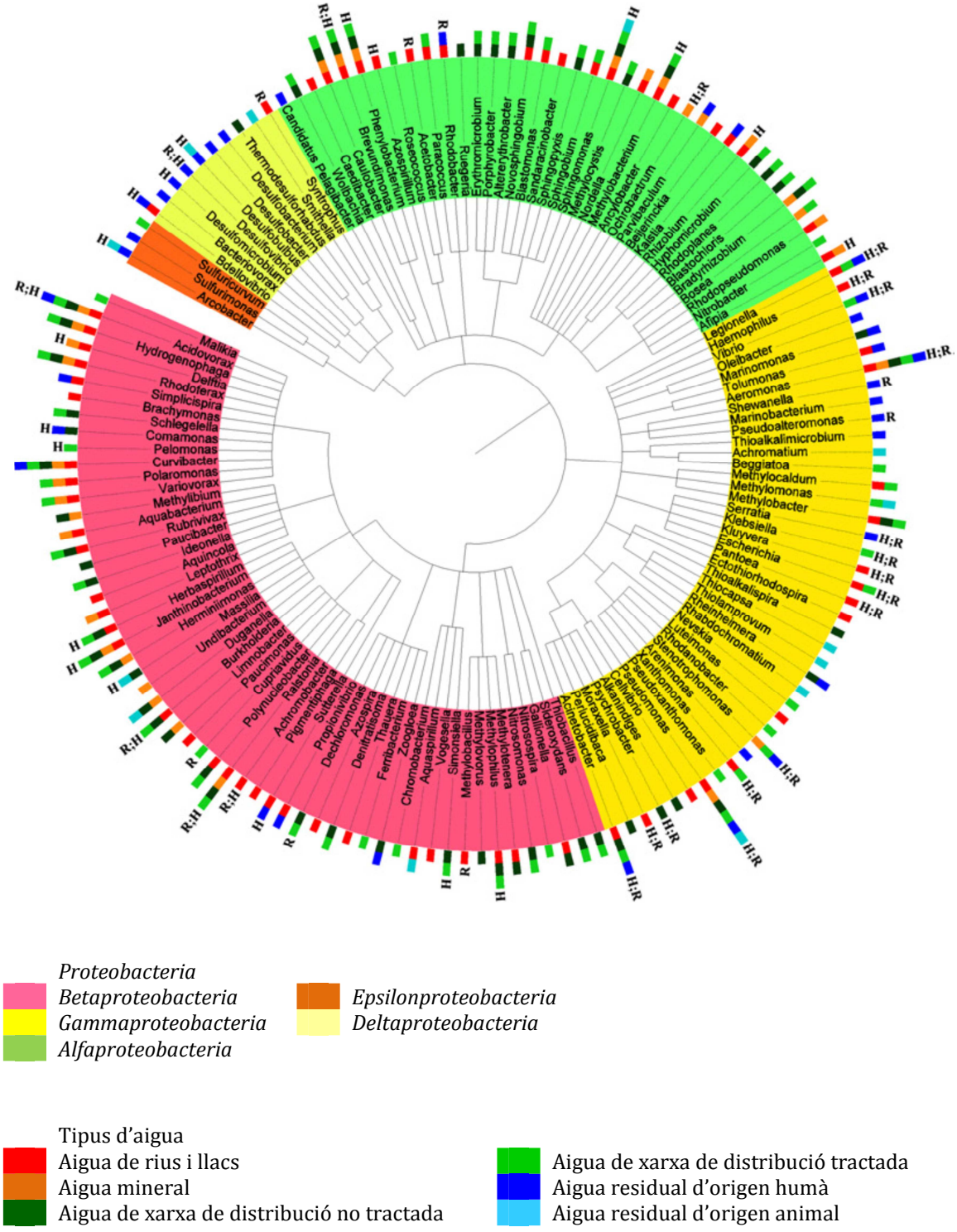
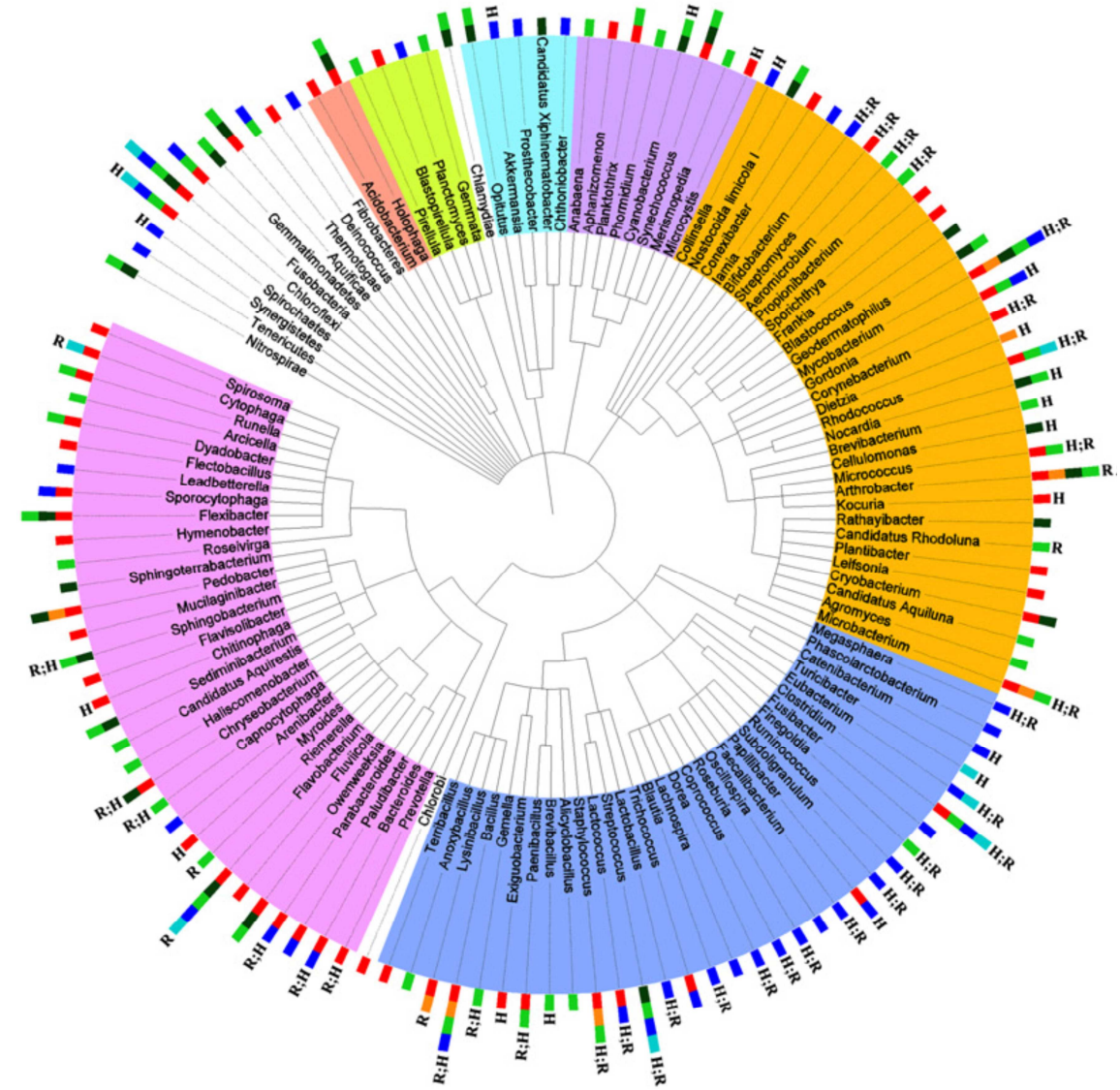


Figura 1a i 1b. Representació de la diversitat bacteriana (a) classes de *Proteobacteria* i (b) altres fílums observats en diferents tipus d'aigua. Cada classe o fílum es representa amb un color diferent. Els requadres de colors de la part externa de la figura indiquen el tipus d'aigua on han estat identificats. H: associat al microbioma humà, R: presenta resistència a antibiòtics. Adapted by permission from Elsevier Ltd, Copyright 2014. License number 3922241294330 (Vaz-Moreira et al., 2014).

1b



Altres fílums
Bacteroidetes
Firmicutes
Actinobacteria
Cyanobacteria
Verrucomicrobia
Planctomycetes
Acidobacteria

Tipus d'aigua
Aigua de rius i llacs
Aigua mineral
Aigua de xarxa de distribució no tractada
Aigua de xarxa de distribució tractada
Aigua residual d'origen humà
Aigua residual d'origen animal

1.1. Aigua mineral natural

1.1.1. Context històric

Edat antiga i medieval

L'aigua ha estat un símbol de purificació, s'ha utilitzat en rituals i en medicina en moltes civilitzacions i religions al llarg de la història de la humanitat (Major, 1954). A l'antiga civilització babilònica, els doctors eren coneguts com els especialistes de l'aigua, se'ls anomenava "azu" que significa coneixedors de l'aigua. Aquests doctors utilitzaven l'aplicació de compreses amb aigua freda o calenta i banys al riu com a part de les seves teràpies. A l'antic Egipte, es venerava el riu Nil que se li atribuïen poders a les seves aigües (Adler, 1993).

Grans santuaris de l'antiga Grècia, com el santuari d'Epidaure i Delfos estaven ubicats en deus, i és que era freqüent la relació entre salut, religió i aigua. De fet, en la mitologia grega trobem diverses referències a l'aigua, per exemple, la deessa Hebe va curar a Mart gràcies a les aigües d'una font. A més, el descobriment de les aigües termals i sulfuroses se li atribueix a Hèrcules. La mitologia narra que després dels treballs feixucs que havia de fer, les aigües li permetien recuperar les forces. Per aquest motiu, les aigües termals van estar sota la protecció d'aquest heroi. De fet, el terme *hercúlia* va ser sinònim de *balnea* (Oró Fernández, 1996).

Heròdot és considerat el fundador de la hidrologia mèdica, ja que en la seva obra ofereix unes directrius per a l'ús de l'aigua mineral i els seus banys per tal de guarir certes malalties (De la Rosa and Mosso, 2004). Hipòcrates, considerat el pare de la medicina, en el seu tractat "*Sobre aires, aigües i llocs"*, estudia l'aigua i de la totalitat de la seva obra es desglossa les propietats positives i negatives que l'aigua ofereix (Oró Fernández, 1996). Posteriorment el metge Asclepíades Bitini va introduir la ingesta d'aigua com a part del seus tractaments tan preventius com curatius, la hidroteràpia. La hidroteràpia va anar prenent importància i molts metges van contribuir fent aportacions en aquesta ciència: classificant les aigües minerals, descrivint les seves propietats curatives... Alguns d'ells van ser: Cels, Dioscòrides, Antilo, Arquigenes, Ruf Efesi, Areteu... i entre aquests, Musa, conegut per haver salvat la vida a l'emperador August d'una hepatitis gràcies als banys d'aigua mineral freda. Aquestes pràctiques van ser adoptades pels romans (Adams, 1886), de fet van ser les úniques teràpies utilitzades pels romans durant 600 anys (De la Rosa and Mosso, 2004). Es van construir moltes termes, algunes ben conegudes; Titus, Constantí i Caracalla. Aquests llocs eren dedicats a la curació diverses malalties (febre, psoriasis,

artritis, cremades) (Jackson, 1990). Galen va proposar els banys de diferents temperatures, principalment aigua freda, com a tractament per a la febre, artritis, refredat... un remei que ha perdurat fins els nostres dies (Panebaker G., 1928). La cultura romana sobre els banys i les termes es va estendre per tot Europa, i es van construir termes a Alemanya, Anglaterra i també els russos i turcs van adoptar aquestes costums (Boogwatson, 1972; Niemineva, 1958). També els pobles celtes, a la Península Ibèrica, seguien un culte a l'aigua a través de les seves fonts que atribuïen el seu poder guaridor a diferents deïtats tutelars (De la Rosa and Mosso, 2004).

Amb la caiguda de l'Imperi Romà i l'ascens del cristianisme, la cultura de les termes va anar en depriment, ja que segons l'església catòlica es considerava infame i immoral (Adler, 1993). Doncs les termes quedaren restringides en monestirs i per malalts greus atribuint la seva cura als sants. La civilització àrab va continuar utilitzant la cultura de l'aigua amb finalitats mèdiques i curatives i també van recuperar moltes termes de la civilització romana que havien quedat en desús. Albucasis, Avicena i Avenzoar, il·lustres metges àrabs recomanaven en sengles obres l'ús de les aigües minerals per diferents malalties (Sánchez Granjel, 1981).

Edat moderna i contemporània

Durant el Renaixement es va difondre l'opinió de metges que relacionaven la utilització de termes amb la transmissió de malalties infeccioses a través de l'aigua. Tot i que després, amb la invenció de la impremta va permetre la ràpida difusió de diverses obres com "*De Balneis et Thermis*" de Savonarola (1498), considerat el primer tractat de Balnoteràpia, *"Cura de la pedra i dolor de la illada i còlic-renal"* de Gutiérrez de Toledo que donen testimoni de l'efecte curatiu de l'aigua (Rodríguez, 1995).

Els descobridors d'Amèrica van observar que les antigues civilitzacions precolombines també aprofitaven l'aigua per a guarir-se, existeixen indicis que les civilitzacions maies també utilitzaven aigües termals (Armijo and San Martín, 1994). Durant els segles XVI i XVII, les classes aristòcrates espanyoles van mostrar interès per les teràpies que oferia l'aigua medicinal, de fet, reis i nobles es feien portar les aigües fins als seus palaus. Nombrosos escriptors com Santa Teresa de Jesús, Lucio Marineo Sículo, Lope de Vega i fins i tot Cervantes a la seva obra "*El Quijote"* elogien les aigües de les fonts espanyoles i de les seves propietats curatives.

Alfonso Limón Montero va publicar a finals del segle XVII l'obra titulada "*Espejo cristalino de las aguas minerales de España"*, considerat el primer tractat d'hidrologia peninsular. En aquesta publicació es descriu la situació geogràfica, les característiques químiques i físiques, els efectes sobre la salut i la forma d'utilització de les diferents aigües minerals d'Espanya. Durant els segles XVII i XVIII, metges i farmacèutics comencen i es sistematitzen els estudis sobre la composició de les aigües minerals (Francés, 1994; Rodríguez, 1995).

En el segle XIX hi havia en funcionament a l'estat espanyol 300 balnearis i més de 1693 deus estaven recollits en el "*Censo General de las aguas en España"*. Les malalties més habituals que es tractaven eren les cròniques com neuràlgies, artritis, reumatismes, malalties de la pell i també infeccions com lepra, tuberculosis, còlera i paludisme. És en aquest moment, als anys 30, en que les aigües minerals comencen a comercialitzar-se per arribar a les llars dels consumidors per tal de prolongar els seus beneficis curatius. Primerament va començar de forma gradual, de 724 deus només s'exportaven 16 (Rubio, 1853), es comercialitzaven a les farmàcies amb ampolles fosques, conegudes com aigües mineromedicinals (Maraver et al., 1998).

A partir dels anys 20 del segle XX, es va consolidar el sector, així en la Guia oficial d'establiments balnearis i aigües medicinals d'Espanya del 1927 es comptabilitzen 161 balnearis i 29 envasadores. Poc a poc canvia la concepció de l'aigua envasada "d'aigua de medicament" a "aigua de taula". Així les aigües envasades comencen a sortir de les farmàcies i entren a comercialitzar-se com un producte d'alimentació (Maraver et al., 1998). Als anys 70, l'aigua envasada va esdevenir "un producte de consum alimentari" d'àmplia distribució. Són diferents motius els que propiciaren aquest canvi. En primer lloc l'increment demogràfic que dificultà l'abastament d'aigua potable de la xarxa en certes zones, l'aparició dels envasos de plàstic més manejables i la valoració de l'aigua mineral com un producte saludable i pur (ANABEA, 2009; Mappa, 2002).

1.1.2. Situació actual del comerç d'aigua mineral natural envasada

La indústria envasadora d'aigua (aigua mineral natural i també d'aigües preparades) ha emergit com un dels sectors industrials més importants a totes les regions del planeta, la comercialització es va iniciar a l'oest d'Europa fins a esdevenir un negoci mundial (Ferrier, 2001). Estats Units va rellevar a Europa com el país amb la indústria envasadora més important al mercat en termes de volum, però darrerament, al 2013, el sector asiàtic ha ocupat la primera posició als mercats i en segueix sent el líder. Empreses locals encara lideren el mercat en certes regions, tot i que, actualment quatre multinacionals dominen el comerç d'aigua envasada mundial.

El consum d'aigua mineral natural embotellada va augmentar significativament fins l'any 2008, coincidint amb l'inici de la gran recessió econòmica mundial. A partir d'aquest moment, la demanda i el consum d'aigua embotellada van disminuir (Rani et al., 2012; Rodwan, 2014; Storey, 2010). El consum mitjà per càpita a l'estat espanyol, l'any 2014 va ascendir als 117 litres, després de 7 anys de recessió degut a la crisi econòmica en que el país ha estat immers. Així, l'estat espanyol es situa com el setè país d'Europa amb el consum més elevat després d'Itàlia, Alemanya, Portugal, Bèlgica, Hongria i França (Fig. 2).

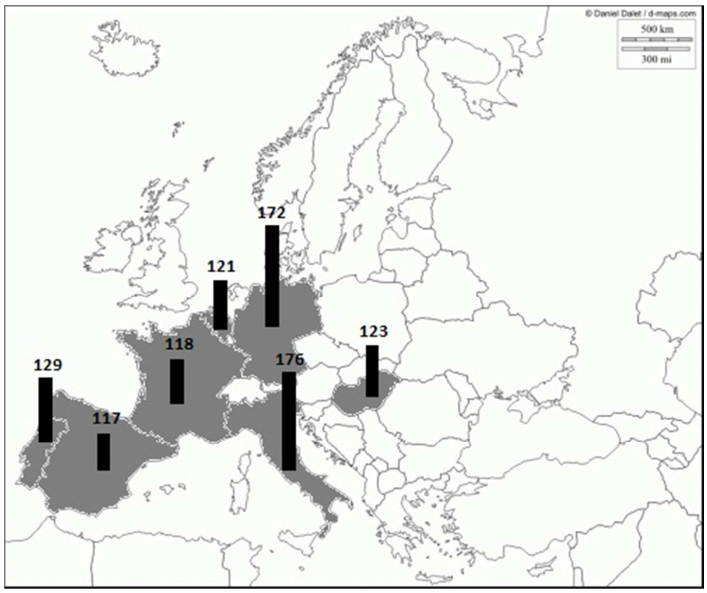


Figura 2. Consum d'aigua envasada en litres per càpita als països de la Unió Europea l'any 2014. Elaboració pròpia a partir de dades de la Federació europea d'aigua envasada (EFBW).

El volum envasat a l'estat espanyol va ser de 5.030 milions de litres a l'any 2014, un 6,5% més respecte l'any anterior segons les xifres de l'Associació nacional d'empreses d'aigües

de beguda envasada (ANEABE) i el primer any amb un increment des del 2006 (Fig. 3). Segons els tipus d'aigua envasada, el 96,5% de la producció correspon a les aigües minerals, el 1,6% a aigües de deu i finalment el percentatge restant correspon a les aigües potables preparades.

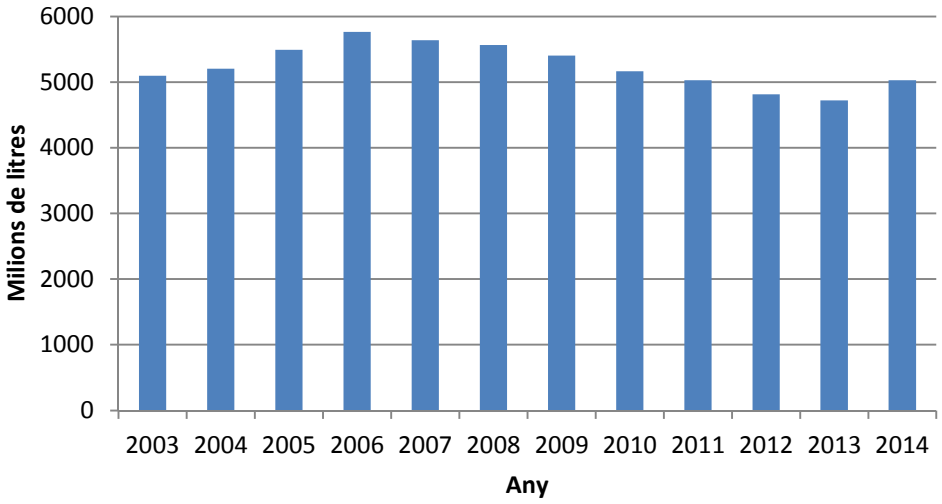


Figura 3. Gràfic sobre l'evolució de la producció d'aigües de beguda envasades a l'estat espanyol en milions de litres. Elaboració pròpia a partir de dades d'ANEABE.

El sector català envasa el 24% del total de litres de l'estat espanyol (1.237 milions de litres a l'any 2014). Pràcticament la gran majoria de les marques catalanes d'aigua mineral natural i de brollador estan agrupades a l'Associació catalana d'envasadors d'aigua (ACEA), que es va constituir l'any 1978.

Segons les prediccions de mercat, el comerç d'aigua envasada pels propers anys s'estima que creixerà i assolirà un altre màxim en el consum per càpita després de la recuperació del moment de debilitat econòmica. Els països de l'Europa de l'Est s'espera que segueixin un model de creixement semblant al que van experimentar els països de l'Europa Occidental a principi dels anys 90. Per contra, el creixement anual serà menor a l'Europa Occidental, ja que el consum anual per persona és elevat. El major increment del mercat de les aigües envasades es preveu principalment a Austràlia i Àsia, i també a Àfrica. En canvi, els països de Sud Amèrica i l'Orient Mitjà s'estima un creixement més moderat (Rani et al., 2012).

L'increment de la demanda d'aigua envasada dels darrers anys juntament amb l'increment que es preveu pels propers anys, pot semblar un hàbit de consum paradoxal de la població. De fet, existeixen molts factors que expliquen la predilecció per l'aigua mineral natural envasada enfront de l'aigua de xarxa de distribució, entre els que podem destacar:

- La insatisfacció per les propietats organolèptiques de l'aigua potable, que afecten el gust i l'olor (Abrahams et al., 2000; Armas and Sutherland, 1999).
- La predilecció per l'aigua amb alta concentració mineral enfront de l'aigua potable amb pocs minerals, tal i com va resultar en un test de sabor a cegues (Doria, 2006; Falahee and MacRae, 1995).
- Una alternativa més saludable envers a altres begudes (Doria, 2006; Rosenberg, 2003).
- Versatilitat de l'envàs, opció de beguda en qualsevol lloc i situació (Rodwan, 2016).
- L'increment econòmic i del benestar de la població que ha facilitat la compra d'aigües embotellades (Doria, 2006).
- Promoció comercial sobre els efectes a la salut de l'aigua mineral (Bharath et al., 2003; Doria, 2006).
- Després d'un brot al sistema de distribució local o en aquells indrets on habitualment existeixen problemes al sistema de distribució d'aigua potable (Doria, 2006).

1.1.3. Marc Normatiu

La Directiva Europea 2009/54/CE de 18 juny de 2009 regula l'explotació i comercialització de les aigües minerals naturals en tots els territoris de la Comunitat Europea. Aquesta directiva derogava la primera regulació comunitària, la Directiva 80/777/CEE de 15 juliol de 1980 relativa a l'aproximació de les legislacions dels Estats membres sobre explotació i comercialització d'aigües minerals naturals, que va ser modificada posteriorment fins a 5 vegades.

El Reial Decret 1074/2002 pel qual es regulava el procés d'elaboració, circulació i comerç d'aigües de beguda envasades va refondre i incorporar a l'ordenament espanyol les directives comunitàries 80/777/CEE i la 98/83/CE relativa a la qualitat de les aigües destinades al consum humà, aquesta última únicament els aspectes referents a l'aigua de beguda envasada. De tal manera que el Reial Decret 1074/2002 regulava tres tipus d'aigües de beguda envasades (aigua mineral natural, de deu i potabilitzada). A fi de garantir una major seguretat jurídica, es va dividir el contingut d'aquesta norma en dues disposicions: el Reial Decret 1798/2010 pel qual es regula les aigües mineral naturals i les aigües de deu envasades pel consum humà i el Reial Decret 1799/2010 pel qual es regula el procés d'elaboració i comercialització d'aigües preparades envasades pel consum humà. La norma vigent a la Comunitat Europea, la Directiva 2009/54/CE va ser transposada a l'ordenament jurídic nacional amb el Reial Decret 1798/2010 (Anonymous, 2011, 2009, 2002, 1998, 1980).

L'aigua mineral natural ha d'ésser originària d'una capa freàtica o jaciment subterrani, que brolli d'una font o pot ser captada artificialment mitjançant sondeig: pou o galeries i microbiològicament saludable, tal i com estableix la Directiva Europea 2009/54/CE.

L'aigua no pot estar sotmesa a cap tractament de desinfecció incloent l'esterilització o la pasteurització, ni la incorporació d'elements bacteriostàtics que puguin modificar el contingut de microorganismes. A excepció dels següents tractaments:

- La filtració o decantació, amb la possibilitat de ser precedida per una oxigenació, per extreure elements no estables com ferro i sulfur, sempre i quan no alteri ni la composició ni les propietats de l'aigua.
- L'eliminació de ferro, manganès, sulfur i arsènic mitjançant un tractament amb aire enriquit d'ozó sempre i quan no alteri ni la composició ni les propietats de l'aigua.
- L'eliminació de diòxid de carboni lliure per mètodes físics exclusivament.

L'aigua mineral natural es distingeix de l'aigua potable per la seva naturalesa que està caracteritzada pel seu contingut en minerals, elements traça i per la seva puresa original, que ha estat preservada intacta gràcies al seu origen subterrani, que l'ha protegit del risc de contaminació. La composició, la temperatura i les característiques essencials de l'aigua mineral natural han de mantenir-se constants, dins dels límits produïts per les fluctuacions naturals. Per tal de preservar les característiques de l'aigua, tot el material utilitzat per l'explotació de l'aigua: sistema d'extracció, canonades, tancs d'emmagatzematge, sistema d'envasat com també l'envàs i els tractaments de neteja que s'apliquin a l'envàs abans del procés d'envasat han de prevenir qualsevol alteració fisicoquímica i microbiològica de l'aigua ni tampoc produir un efecte advers a la microbiota de l'aigua.

Al brollar de la font, el total de colònies no pot superar 20 UFC/ml a 20-22 ˚C a 72 hores i 5 UFC/ml a 37 ˚C després de 24 hores. De fet, aquestes concentracions no són valors màxims permesos, sinó són valors orientatius, ja que el recompte al brollar de la font s'ha d'ajustar als recomptes totals de colònies que s'obtenen dels controls periòdics a la font sempre que hi hagi evidència que la font està protegida de qualsevol contaminació.

Després de l'envasat, el total de colònies no pot superar 100 UFC/ml a 20-22 ˚C a 72 hores, ni 20 UFC/ml a 37 ˚C transcorregudes 24 hores. Les ampolles s'han d'analitzar abans de les 12 hores d'haver estat embotellades i durant aquest període s'han de mantenir a 4 ˚C.

A la font i durant la seva comercialització, l'aigua ha d'ésser lliure de paràsits i microorganismes patògens:

- Coliforms i enterocs fecals, absència en 250 ml.
- *Escherichia coli,* absència en 250 ml.
- Espores de sulfit reductors anaeròbics, absència en 50 ml.
- *Pseudomonas aeruginosa,* absència en 250 ml.

Durant la comercialització, el contingut de microorganismes revivificables ha d'ésser el resultat de l'evolució normal d'aquella població bacteriana present a la font. També l'aigua mineral no ha contenir canvis organolèptics. Per aquest motiu, tot recipient utilitzat per a l'envasat d'aigua mineral natural haurà d'estar equipat amb dispositius de tancament dissenyats per evitar qualsevol possibilitat de contaminació o d'adulteració del producte.

El Reial Decret 1798/2010 també regula les aigües de deu, a diferència de la normativa comunitària 2009/54/CE que només fa esmena a les aigües minerals naturals. Les aigües de deu, a diferència de les aigües minerals, poden mantenir constant o no la seva composició química (contingut en minerals i oligoelements). Les aigües de deu han de complir els mateixos requisits microbiològics esmentats anteriorment.

1.2. Aigua de xarxa de distribució

1.2.1. Context històric de l'aigua de xarxa de distribució a l'àrea de Barcelona

A la primera dècada del segle XX, als països industrialitzats s'implantà la potabilització de l'aigua per filtració a través dels llits de sorra i l'aplicació de clor o compostos clorats per a la desinfecció. A Catalunya, la potabilització de l'aigua no va ser introduïda fins a la segona dècada del segle XX. La primera mostra de desinfecció química de l'aigua d'abastament a Catalunya va ser a l'any 1914 durant l'epidèmia de la febre tifoide a Barcelona (Jofre, 2010). A mitjans del segle XX, Barcelona i rodalies s'abastien exclusivament d'aigua subterrània, provinent dels pous situats al Besòs i al Llobregat. La demanda de l'aigua va incrementar als anys 40, degut a la forta industrialització i l'increment demogràfic de l'àrea, així que l'aigua subterrània no va ser suficient per cobrir totes les necessitats. Per tal d'atendre la demanda va ser indispensable recórrer a l'aprofitament de l'aigua superficial del riu Llobregat, per aquest motiu es va sol·licitar una concessió per l'ús de l'aigua l'any 1953 i també per la construcció d'una Estació de tractament d'aigua potable (ETAP) a Sant Joan Despí (Aigües-Barcelona, 2010).

1.2.2. ETAP Sant Joan Despí

L'ETAP de Sant Joan Despí s'inaugurà l'any 1955 amb la finalitat de subministrar aigua potable amb totes les garanties sanitàries a diverses ciutats de l'Àrea metropolitana de Barcelona (Fig. 4).

Figura 4. Vista aèria de l'ETAP de Sant Joan Despí. Font: Aigües Barcelona.

L'ETAP de Sant Joan Despí és una de les plantes més completes d'Europa per la tecnologia que aplica en el tractament de l'aigua. A més dels processos de pretractaments, clarificació i desinfecció final, incorpora un tractament de l'aigua amb ozó i carbó actiu, que assegura

una millor qualitat. L'any 2009 s'instal·là el tractament mitjançant membranes d'ultrafiltració i osmosi inversa. L'ETAP potabilitza l'aigua del riu Llobregat i les aigües subterrànies de l'aqüífer de la vall baixa del riu, la planta permet potabilitzar cabals de fins a 5.300 litres per segon i al llarg de l'any tracta 187 hm^3/any d'aigua. Actualment la planta abasteix a 21 municipis de l'Àrea metropolitana de Barcelona, un total de 1.200.000 habitants.

1.2.3. Descripció del procés de potabilització de l'aigua

L'aigua del llit del riu Llobregat és captada a través d'unes reixes, formades per un conjunt de barres paral·leles de formigó armat que condueixen l'aigua a través d'unes galeries a l'interior de l'ETAP fins a l'etapa de desarenament. Aquesta etapa té com a finalitat l'eliminació de materials gruixuts, graves i sorres principalment. La secció del canal de conducció s'eixampla, com a conseqüència es produeix una pèrdua de velocitat que afavoreix la precipitació de les sorres. Seguidament l'aigua és bombada i amb la càrrega hidràulica proporcionada possibilita la circulació per gravetat per les fases del pretractament que consten d'una floculació, sedimentació i filtració a través de filtres de sorra (Fig. 5) (Aigües-Barcelona, 2010).

Primerament s'afegeix coagulant (sals d'alumini) i floculant (clorur fèrric), per tal d'aglutinar les partícules presents i diòxid de clor. Aquest compost actua com agent desinfectant i oxida cert metalls com el ferro, el manganès i la matèria orgànica. Per a la sedimentació del material coagulat, l'aigua travessa uns tancs de forma tronc piramidal realitzant un recorregut ascendent. Els tancs de sedimentació treballen mantenint un llit de fangs estabilitzat, perquè els nous flòculs s'uneixin amb els preexistents i sedimentin. Per tal d'eliminar els flocs més petits que no hagin sedimentat, l'aigua es filtra per gravetat a través dels filtres de sorra i és recollida al fons per uns col·lectors. El llit filtrant està format per diverses capes de sorres i graves de granulometria diversa, a més a més, permet eliminar patògens com *Giardia* sp. i *Cryptosporidium* sp., que queden retinguts per la seva grandària. En aquest punt del procés de tractament, l'aigua subterrània de l'aqüífer del Llobregat és incorporada. L'aigua s'eleva una segona vegada per proporcionar l'energia suficient per les fases de tractament. En aquest punt, l'aigua es divideix en dues línies de tractament independents: una fracció de l'aigua se li aplica un tractament d'ozonització i de filtració per carbó actiu i l'altra fracció un tractament per membranes (ultrafiltració i osmosi inversa).

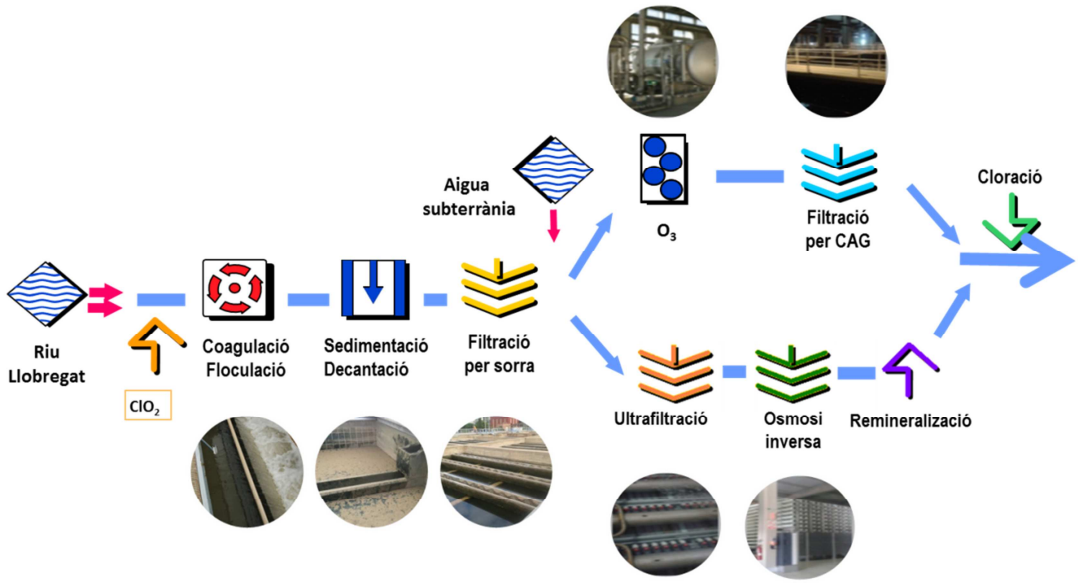


Figura 5. Esquema del procés de potabilització de l'aigua a l'ETAP de Sant Joan Despí. Font: Aigües Barcelona.

La primera línia de tractament (Fig. 5) s'addiciona ozó mitjançant difusors porosos en les cambres de contacte per on circula l'aigua. L'ozó permet oxidar la matèria orgànica i eliminar els microorganismes, ja que té efectes biocides i oxidants. A continuació, els filtres de carbó actiu granular (CAG) adsorbeixen els compostos orgànics i també retenen òxids metàl·lics (ferro, manganès, níquel...). Aquests dos tractaments permeten millorar les característiques organolèptiques de l'aigua.

La segona línia de tractament (Fig. 5) consta d'una ultrafiltració, abans d'entrar a les cambres on estan submergits els trens de membranes d'ultrafiltració és necessari una acidificació de l'aigua, que es realitza amb l'addició àcid sulfúric per tal de maximitzar la retenció de l'alumini residual. Les membranes d'ultrafiltració treballen submergides i aspiren l'aigua cap al seu interior, per la mida dels seu porus permeten la retenció de material en suspensió i dels bacteris. L'aigua, abans d'entrar als bastidors d'osmosi inversa (OI) requereix un pretractament per tal de protegir les membranes d'osmosi. Aquest pretractament consta de radiació ultraviolada, addició d'àcid sulfúric per ajustar el pH de l'aigua, per evitar precipitacions a les membranes, addició de bisulfit per tal d'eliminar restes d'oxidants i un dispersant que alenteix els fenòmens de cristal·lització de les sals de l'aigua. Seguidament s'aplica una filtració de cartutx, i altra vegada radiació ultraviolada per eliminar bacteris i virus, i conferir una protecció addicional. La planta d'osmosi inversa està formada per 10 bastidors, cada bastidor consta de 158 tubs i cada tub conté 7 membranes d'osmosi inversa, el que fan un total de 11.060 membranes d'osmosi inversa.

El cabal d'alimentació per a cada bastidor és de 265 l/s. Cada bastidor consta de 3 etapes que poden recuperar entre un 85 i un 90% de l'aigua d'entrada. Un bombament a alta pressió (8-16 bar) permet el pas de l'aigua per les membranes d'OI. L'aigua osmotitzada requereix una remineralització ja que l'aigua amb poc contingut de sals té propietats agressives i incrustants. La remineralització es realitza per contacte amb les cambres dels llits de calcita ($CaCO_3$).

Les dues línies de tractament convergeixen en una cambra de mescla on té lloc la cloració que eliminarà el contingut d'amoni que roman a l'aigua després del tractament d'ozonització-filtració per carbó actiu i garantirà la desinfecció. A continuació, l'aigua és conduïda a un altre dipòsit on s'efectua la cloració final, per a evitar la formació de zones mortes, l'aigua segueix un recorregut laberíntic, presentant un temps de retenció de 30 minuts. Finalitzat el tractament de potabilització, l'aigua és distribuïda en dos dipòsits, amb una capacitat de 4.000 m^3, que alimenten les estacions de bombament de la xarxa de distribució.

1.2.3.1. Osmosi inversa

L'objectiu de l'aplicació de la tecnologia de membranes d'osmosi inversa en una planta de tractament d'aigua és l'obtenció d'aigua d'alta qualitat. L'OI és un procés de separació de membrana per pressió mitjançant el qual una membrana semipermeable rebutja els constituents dissolts a l'aigua d'alimentació, però permet el pas de l'aigua. L'OI elimina de manera eficient una àmplia varietat de contaminants de l'aigua: components microbians i compostos tant orgànics com inorgànics (Bereschenko et al., 2010). La implantació d'aquesta tecnologia va lligada amb el desenvolupament de les membranes ja que aquestes determinen l'eficiència tècnica i del rendiment econòmic del procés (Kang and Cao, 2012).

Figura 6. Imatge de la planta d'osmosi inversa de l'ETAP de Sant Joan Despí (Aigües Barcelona). Elaboració pròpia.

En els darrers anys, les membranes d'OI han estat àmpliament implantades com a part del procés de potabilització d'aigua, no obstant això, la principal limitació d'aquesta tecnologia és el deteriorament de les membranes (*fouling*). El *fouling* és un procés complex que implica la deposició de soluts (inorgànics i/o orgànics) o partícules de l'aigua d'alimentació juntament amb incrustacions biològiques (*biofouling*). El *biofouling* consisteix en la deposició de material biològic i la formació de biopel·lícules (biofilms) (Herzberg and Elimelech, 2007; Kang and Cao, 2012). El *fouling* provoca un increment de l'energia necessària pel bombeig de l'aigua d'entrada, un increment de la freqüència de manteniment de les membranes i una disminució del flux i de la qualitat del producte (Bereschenko et al., 2008; Chon et al., 2012; Flemming, 2002). Actualment s'està treballant en el desenvolupament de noves membranes per tal d'evitar la formació de *fouling* i incrementar el temps de vida de les membranes. Encara que aquestes propostes estan en fase de desenvolupament es basen en la modificació de la superfície de les membranes (introduint una capa hidròfila, reduint la rugositat i fomentant la repulsió electrostàtica) i en el desenvolupament de nous materials (Kang and Cao, 2012).

Figura 7. Imatge d'un tub amb les membranes d'osmosi inversa a l'interior. Elaboració pròpia, a l'ETAP de Sant Joan Despí (Aigües Barcelona).

El procés de formació de biofilm ha rebut especial atenció en els darrers anys, ja que els bacteris que en formen part són més resistents a la desinfecció que no en el seu estat planctònic, gràcies als exopolímers que es sintetitzen i que protegeixen físicament als bacteris (Costerton et al., 1995). Les primeres poblacions que formen el biofilm són estocàstiques, provinents del reclutament d'individus del medi, després pot estar controlat per circuits de comunicació intra i intercel·lular, anomenats circuits de *quorum sensing*. Aquests són activats per la presència de certes molècules senyal o autoinductors que els propis bacteris excreten en resposta a la densitat de població cel·lular. Aquestes molècules serveixen de senyal química per induir l'expressió genètica col·lectiva (Miller and Bassler, 2003). En el moment que la població és més abundant, s'incrementa la competitivitat pels nutrients, desplaçant així aquells microorganismes menys competitius, així que en la fase de desenvolupament del biofilm, la diversitat decreix. En canvi, la diversitat d'un biofilm

madur incrementa, hi ha una diversificació dels nínxols i un reciclatge intern de nutrients (Besemer et al., 2007; Jackson et al., 2001; Lyautey et al., 2005).

Els biofilms que contribueixen al deteriorament de les membranes d'OI i que també es desenvolupen en els conductes de la planta i en els sistemes de distribució d'aigua poden estar associats amb riscos per a la salut humana, ja que poden albergar i ser reservoris de patògens oportunistes. Alguns d'aquests patògens aïllats de biopel·lícules en els sistemes de distribució d'aigua potable inclouen *Mycobacterium avium, Pseudomonas aeruginosa, Klebsiella* spp., *Legionella* spp. i *Flavobacterium* spp. (Norton and LeChevallier, 2000; Ridgway and Olson, 1981; Walker et al., 1993).

Entre els patògens oportunistes més recurrents, *Pseudomonas aeruginosa* causa infeccions greus i persistents en individus immunocompromesos i que pateixen fibrosi quística. Aquesta espècie està associada a malalties de transmissió hídrica i alimentària (Römling et al., 1994; Warburton, 1993). *P. aeruginosa* es troba en molts ambients, té la capacitat de créixer amb baixes concentracions de nutrients (González et al., 1987; Moreira et al., 1994). Aquesta espècie pot jugar un paper important en les primeres fases de la formació de biofilm, gràcies a la motilitat mitjançant el pili tipus IV "*twitching*" (O'Toole et al., 2000; Wall and Kaiser, 1999), a més a més, el *quorum sensing* en *P. aeruginosa* regula la síntesis de factors de virulència i factors de resposta a l'estrès (Lee and Zhang, 2015). *Pseudomonas* spp. s'ha proposat com un bon indicador de recreixement en sistemes de distribució d'aigua, especialment a Catalunya (Ribas et al., 2000).

1.2.4. Marc Normatiu

La Directiva Europea 98/83/CE relativa a la qualitat de les aigües destinades al consum humà és la normativa comunitària vigent actualment que regula l'aigua de distribució i s'articula a l'estat espanyol a través Reial Decret 140/2003 de 7 de febrer pel qual s'estableixen els criteris sanitaris de la qualitat de l'aigua de consum humà. Aquesta Directiva 98/83/CE va substituir l'anterior normativa (Directiva 80/777/CEE relativa a la qualitat de les aigües destinades pel consum humà) i en va adaptar el contingut considerant els progressos científics i tècnics que s'havien produït. Darrerament la Directiva 98/83/CE va ser modificada per la Directiva 2015/1787/CE per la qual es modifiquen els annexos II i III, aquestes modificacions dels annexos pretenen actualitzar la normativa amb els darrers progressos científics i tècnics i les condicions específiques per realitzar el control d'autories i el control de comprovació que a l'anterior disposició permetien un cert grau de flexibilitat en l'aplicació d'elles (Anonymous, 2015, 2003, 1998, 1980). A efectes de la disposició s'entén per aigua de consum humà i regulades per la present normativa:

- Totes les aigües utilitzades per beure, cuinar, preparar aliments, la higiene personal o altres usos domèstics, independent del seu origen i que es subministrin al consumidor a través de xarxes de distribució públiques o privades, de cisternes i de dipòsits públics o privats.
- Totes les aigües utilitzades a la indústria alimentària per a qualsevol finalitat així com les utilitzades per a la neteja de les superfícies, objectes i materials que puguin estar en contacte amb els aliments.
- Totes les aigües subministrades pel consum humà com a part d'una activitat comercial o pública.

L'aigua destinada al consum humà, als efectes del Reial Decret 140/2003 pot tenir qualsevol origen, sempre que no comporti un risc per a la població. La dotació diària per càpita per tal de cobrir les necessitats higièniques-sanitàries de la població i el desenvolupament de l'activitat de la zona es fixa com a 100 litres per habitant i dia segons el Reial Decret 140/2003. L'aigua de consum humà ha d'ésser salubre i neta, per tant segons la normativa implica que no pot contenir cap tipus de microorganisme, paràsit o substància, en una quantitat o concentració que pugui suposar un risc per a la salut humana. També ha de complir els següents paràmetres microbiològics i químics (els

paràmetres químics no han estat llistats, consultar Reial Decret 140/2003, annex I, part B):

- *Escherichia coli,* absència en 100 ml.
- Enterococs, absència en 100 ml.
- *Clostridium perfringens* (incloses les espores), absència en 100 ml. Quan la mostra sigui positiva per *Clostridium perfringens* i tingui una tèrboles superior a 5 UNF s'ha de determinar també cryptosporidium.

L'aigua de consum humà distribuïda a la població a través de xarxes de distribució ha de ser desinfectada, els subproductes derivats de la desinfecció han de tenir els nivells més baixos possibles, sense comprometre l'eficàcia de la desinfecció. La xarxa de distribució ha de ser de disseny mallat per a eliminar punts que facilitin la contaminació, i davant de situacions anòmales han de disposar dels mecanismes per tal d'aïllar els sectors afectats.

El control de l'aigua de consum es responsabilitat del gestor, els punts de mostreig han de ser representatius de la xarxa de distribució i els ha de fixar el gestor amb la supervisió de l'autoritat sanitària. El tipus d'anàlisi microbiològica que s'ha d'aplicar és:

- *Escherichia coli,* absència en 100 ml.
- Enterococs, absència en 100 ml.
- *Clostridium perfringens* (incloses les espores), absència en 100 ml.
- Microorganismes coliformes, absència en 100 ml.
- Recompte de colònies a 22 ˚C:
    - A la sortida de l'ETAP: 100 UFC/ml a 22 ˚C.
    - A la xarxa de distribució, el recompte de colònies a 22 ˚C no ha de presentar canvis anòmals.

1.3. Estandardització dels paràmetres microbiològics

La Directiva 2015/1787/CE especifica els mètodes d'anàlisi dels diferents paràmetres microbiològics per a les aigües, tots ells validats i acceptats per l'Organització internacional d'estandardització (ISO) que ha desenvolupat un seguit de normes per tal d'estandarditzar i harmonitzar els diferents protocols existents (Anonymous, 2015).

- *Escherichia coli* i microorganismes coliforms: ISO 9308-1:2014 Detecció i enumeració de coliforms i *E. coli* — Part 1: Mètode de filtració per membrana (ISO, 2014) o ISO 9308-2:2012 Detecció i enumeració de coliforms i *E. coli* — Part 2: Mètode del número més probable (ISO, 2012).
- *Enterococcus:* ISO 7899-2:2000 Detecció i enumeració d'enterococs intestinals — Part 2: Mètode de filtració per membrana (ISO, 2000).
- *Clostridium perfringens:* ISO 14189:2013 Enumeració de *Clostridium perfringens* — Mitjançant filtració per membrana (ISO, 2013).
- *Pseudomonas aeruginosa:* ISO 16266:2006 Detecció i enumeració de *Pseudomonas aeruginosa* — Mètode de filtració per membrana (ISO, 2006).
- Enumeració de microorganismes cultivables, recompte de colònies a 22 ˚C i 37 ˚C: ISO 6222:1999 Enumeració de microorganismes cultivables — Recompte de colònies per inoculació a medi agar nutritiu (ISO, 1999).

És essencial disposar de protocols acurats i solvents atès que tota millora en el sistema de detecció d'aquests microorganismes patògens i/o indicadors de contaminació fecal, reverteix directament en una major qualitat i seguretat de les aigües de consum.

1.4. Les comunitats heteròtrofes de l'aigua

Robert Koch va publicar un article titulat "*About detection methods for microorganisms in water*" l'any 1883, on introdueix l'ús d'indicadors microbians per a la vigilància de la qualitat de l'aigua i descriu per primera vegada la metodologia per a quantificar el nombre de colònies de bacteris heteròtrofs en placa, *heterotrophic plate count* (HPC) en anglès (Bartram et al., 2003).

Els microorganismes heteròtrofs són tots aquells que requereixen carboni orgànic pel seu creixement. Aquest tipus de microorganismes es troben en l'aigua, el sòl, la vegetació i els aliments. Dins d'aquest grup s'inclouen bacteris, llevats i floridures. El terme HPC es refereix a tots els microorganismes heteròtrofs que poden créixer per un mètode en particular amb unes variables determinades: temperatura d'incubació, temps i medi de cultiu. No tots els microorganismes metabòlicament actius a l'aigua podran créixer en unes condicions de cultiu determinades, sinó que la població que es desenvoluparà difereix en funció de la metodologia utilitzada. En definitiva, les condicions de cultiu tenen un efecte sobre quins microorganismes podran créixer (Allen et al., 2004; Bartram et al., 2003).

En ambients aquàtics, els medis amb concentracions baixes de nutrients afavoreixen que una major diversitat d'heteròtrofs creixin en placa. El medi de cultiu R2A es va dissenyar específicament per a recuperar microorganismes de sistemes aquàtics. Aquest medi conté baixes concentracions de nutrients i baixa força iònica (Allen et al., 2004; Reasoner, 1990). Incubacions llargues entre 5 i 7 dies i a baixes temperatures 20 °C–28 °C afavoreixen la recuperació d'un nombre més elevat d'heteròtrofs en aigües (Reasoner, 1990). Els microorganismes patògens també creixen en els medis dissenyats per recomptes heteròtrofs, i és necessari l'ús de medis selectius per tal de diferenciar-los (Allen et al., 2004).

Encara que existeixen moltes metodologies per a determinar els HPC, hi ha un mètode estandarditzat per l'ISO i recollit a la normativa, la norma ISO 6222:1999 (ISO, 1999), un procediment basat en la utilització del medi agar extracte de llevat, sembra per massa i incubació a 22 °C i 37 °C durant 72 h i 48 h respectivament.

Un estudi realitzat per Gensberger i col·laboradors (Gensberger et al., 2015) posa de manifest les diferències que s'obtenen en la recuperació de la comunitat heteròtrofa d'una

aigua de pou en funció de les condicions d'aïllament fixades. En aquest treball es va comparar la metodologia establerta per l'ISO, que determina un medi de cultiu ric, amb un medi de cultiu baix en nutrients, R2A, recomanat en les normes de la *Environmental protection agency* (EPA) d'Estats Units per a l'aïllament de comunitats heteròtrofes en dues temperatures diferents. L'estudi va revelar que existien diferències significatives en la composició d'heteròtrofs obtinguts degut a la temperatura però no segons el medi de cultiu utilitzat, tot i que l'abundància de certes espècies era diferent i l'índex de diversitat de Simpson va ser major en el medi R2A (Fig. 8).

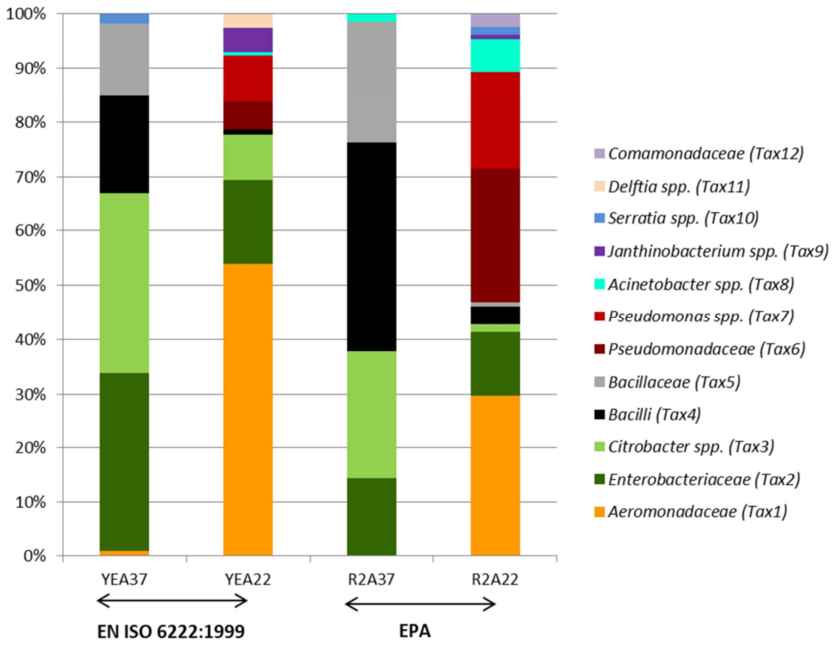


Figura 8. Identificació de la composició de la comunitat a partir de la seqüenciació parcial del gen ARNr 16S de les soques bacterianes aïllades segons les recomanacions ISO i EPA. Medis utilitzats: YEA (Extracte de llevat agar) i R2A. Temperatura d'incubació: 22 ˚C i 37 ˚C. Font: (Gensberger et al., 2015).

El recompte d'heteròtrofs conjuntament amb els indicadors de contaminació fecal *(*coliforms fecals*, Escherichia coli, Enterococcus faecalis,* espores de sulfit reductors, colífags somàtics i bacteriòfags de *Bacteroides*) són els criteris utilitzats per a l'avaluació de la qualitat de l'aigua. En absència d'indicadors de la contaminació fecal, no existeixen evidències clíniques ni epidemiològiques que relacionin concentracions altes HPC amb efectes sobre la salut humana (Allen et al., 2004). Tot i que no hi ha una relació directa sobre la salut, s'ha establert un valor màxim de HPC en les diferents normatives sobre la qualitat de l'aigua, el qual difereix segons el país. Habitualment, la concentració màxima

acceptada és de 500 UFC/ml, aquesta concentració es va establir, no per un risc sobre la salut humana, sinó perquè concentracions superiors interfereixen en la detecció de coliforms fecals, produint valors negatius en els medis de cultiu basats en la lactosa (Edberg et al., 2000).

La majoria dels microorganismes que habiten l'aigua subterrània són heteròtrofs, capaços de subsistir en condicions oligotròfiques (baixes concentracions de matèria orgànica). Els aqüífers es caracteritzen per tenir la taxa de flux constant i la composició química estable com també la temperatura. Les comunitats bacterianes en cada estrat geològic poden ser diferents, es creu que aquestes comunitats procedeixen del transport a través del flux hidrològic des de la superfície. L'estratificació de les comunitats bacterianes pot ser deguda a colonitzacions horitzontals i verticals dirigides pel flux hidrològic (Leclerc and da Costa, 2011; Madsen and Ghiorse, 1993). L'abundància dels bacteris disminueix amb la profunditat, seguint el gradient de nutrients i de disponibilitat d'oxigen (Leclerc and da Costa, 2011). Les zones de recàrrega de nutrients i del transport de l'oxigen alberguen una població bacteriana més nombrosa, aquestes zones són la part més superficial i la zona d'interfase entre la zona saturada i insaturada de l'aqüífer. La major part dels microorganismes es troben adherits a les partícules del sòl, per tant els microorganismes que viuen de forma lliure poden ser diferents dels units a les partícules (Madsen and Ghiorse, 1993).

La font de carboni en els aqüífers depèn del carboni orgànic dissolt (DOC) transportat pel flux hidrològic o de components orgànics que provenen de la sedimentació de material a la superfície que posteriorment són degradats. Als estrats superiors, la concentració de carboni orgànic dissolt és inferior ja que es requereix un temps per a degradar la matèria orgànica, la màxima concentració de DOC s'assoleix en la zona intermèdia de l'aqüífer, on la concentració de DOC és de mitjana de 0,1 a 0,7 mg/l (Morita, 1998). Els microorganismes heteròtrofs dominen la comunitat, els eucariotes són absents o en baixes concentracions, algues i cianobacteris no són presents ja que en els estrats la fotosíntesis no té lloc.

Els bacteris heteròtrofs han desenvolupat mecanismes per resistir períodes llargs sense nutrients, doncs molts microorganismes es troben en un estat de latència, que és un estat fisiològic en que no té lloc el creixement ni la divisió cel·lular per la poca disponibilitat de nutrients (Morita, 1982). Els microorganismes responen en aquest estat de latència per mitjà de diferents mecanismes: formant espores o cists, és a dir diferents tipus de formes

de resistència, modificant el seu metabolisme mantenint un metabolisme basal (Matin, 1992) i expressant sistemes de transport i altres rutes metabòliques per a la utilització d'altres nutrients (Morita, 1998; Nyström, 1993). Les cèl·lules en un estat de latència tenen un mida més petita de l'habitual, així la relació superfície/volum és major permetent la captació de nutrients més eficientment (Morita, 1998). Aquestes microcèl·lules poden passar a través dels filtres de 0,2 μm.

L'estudi de les comunitats es realitza mitjançant l'extracció per bombeig des de l'aqüífer, però aquest mètode pot presentar problemes com la contaminació durant el procés i així dificultar la diferenciació de la microbiota autòctona i al·lòctona. A més, la perforació del terreny modifica les condicions ambientals dels aqüífers, i també pot produir canvis en les comunitats.

1.4.1. Les comunitats microbianes de les aigües minerals naturals

El nombre de microorganismes cultivables en el punt de surgència és baix, aproximadament 10 UFC/ml (Bischofberger et al., 1990; França et al., 2014), però després l'embotellament, experimenten un augment de fins a 10^4-10^5 UFC/ml durant les dues primeres setmanes (Falcone-Dias and Farache Filho, 2013; Leclerc and Moreau, 2002; Urmeneta et al., 2000). Un cop assolida la concentració màxima de bacteris, generalment romanen constants o disminueixen lleugerament durant el primer any després de l'envasat (Bischofberger et al., 1990; Sefcova, 1997).

Primerament, aquest fenomen de creixement exponencial després de l'envasat es va atribuir a "l'efecte ampolla", que en funció de la relació entre l'àrea superficial i el volum de l'ampolla promovia el creixement bacterià. Major superfície en relació al volum, la població bacteriana incrementava més ràpidament, doncs l'efecte ampolla implicava que en ampolles petites, el creixement bacterià fos major. La hipòtesis es fonamentava en que la matèria orgànica era absorbida a la superfície de l'envàs i així esdevenia més accessible als microorganismes. L'efecte ampolla va ser documentat per primera vegada per Buttiaux i Boudier (Buttiaux and Boudier, 1960) i corroborat posteriorment per diversos estudis (Morais and Costa, 1990; Warburton, 1993). Posteriorment, es va negar aquesta hipòtesi, Hammes i col·laboradors, en el seu estudi van utilitzar diferents mides d'ampolles i no van

trobar evidències de la relació superfície/volum amb la concentració bacteriana (Hammes et al., 2010).

Els factors que expliquen l'increment de la població bacteriana encara no són ben coneguts, però s'ha plantejat que el procés d'envasat que produeix un augment de la temperatura i de l'oxigenació de l'aigua, i la posterior exposició a la llum solar i el contacte amb el material de l'ampolla poden estimular el creixement de certes poblacions bacterianes. També s'ha suggerit que la concentració de la matèria orgànica disponible pugui ser utilitzats com a material per a la multiplicació dels microorganismes (Falcone-Dias and Farache Filho, 2013). De fet, hi ha una clara correlació entre el nombre HPC i la concentració de carboni orgànic assimilable (AOC) de l'aigua mineral a les ampolles (Diduch et al., 2016). El nou creixement bacterià també podria explicar-se com a resultat de la reactivació de les cèl·lules en estat de latència inicialment presents a les aigües de l'aqüífer i que es troben en un estat de viabilitat però que no són cultivables (Leclerc and Moreau, 2002).

Els recomptes de microorganismes en les aigües minerals són basats en mostres directes de l'aigua de dins l'ampolla. Ara bé, cal tenir en compte que en les aigües minerals pot coexistir dos tipus de microbiota: una suspesa a l'aigua i una unida a la superfície interior de l'ampolla i que existeixi una dinàmica entre elles. En l'estudi de Jayasekara i col·laboradors, la població adherida a la superfície diferia entre ampolles, en algunes ampolles entre un 5% fins a un 44% de la població es trobava adherida a les parets (Jayasekara et al., 1999). No obstant, la concentració de la població adherida era insuficient per a constituir biofilm, ja que generalment és necessari 10^9 cèl·lules/cm^2 per establir el biofilm (LeChevallier et al., 1987). En observacions de la microbiota de la superfície amb el microscòpic SEM es van observar densitats de 10^7 cèl·lules/cm^2 però la biomassa no era confluent i per tant sense biofilm visible. Alguns microorganismes s'adhereixen amb més força que d'altres segons la seva hidrofobicitat cel·lular o la producció de mucílag. Per tant, aquestes característiques varien en funció de les espècies. Per exemple, s'ha observat que *Brevundimonas vesicularis, Acidovorax delafieldii* i *Methylobacterium mesophilicum* tenen més facilitat per a unir-se a les parets de clorur de polivinil (PVC) (Jayasekara et al., 1999).

Actualment, les ampolles de tereftalat de polietilè (PET) són les més utilitzades per a l'envasat de l'aigua mineral i han reemplaçat les ampolles de vidre, que han quedat reclutades principalment al sector de la restauració i l'hostaleria. El PET, gràcies a les

seves propietats, ofereix un gran nombre d'avantatges: baix pes, transparència, flexibilitat i resistència als impactes (Spangenberg and Vennemann, 2008; Welle, 2011). El material de l'envàs podria afectar el desenvolupament de les poblacions bacterianes, però fins a la data d'avui, molt pocs estudis han avaluat l'efecte del material de l'ampolla a les comunitats bacterianes. Alguns estudis van revelar que les substàncies de baix pes molecular que migren del plàstic poden promoure el creixement de les comunitats. En canvi, els productes residuals de neteja, si les ampolles es reutilitzen, poden interferir en les comunitats bacterianes, produint un efecte bacteriostàtic (Bischofberger et al., 1990). A més, el color del material de l'envàs implica un desenvolupament diferents de les comunitats. Així menys HPC es van obtenir en ampolles de PVC transparents en comparació amb ampolles de vidre fosc, on el color de l'ampolla podria protegir els bacteris de la llum solar (Mavridou, 1992).

Les fonts i aqüífers posseeixen una microbiota pròpia que les caracteritza i diferencia, per tant les aigües minerals no són estèrils, sinó uns ecosistemes complexos amb molta diversitat fenotípica i genètica (Casanovas-Massana and Blanch, 2012; Falcone-Dias and Farache Filho, 2013; Leclerc and Moreau, 2002; Rosenberg, 2003). Els microorganismes aïllats més habitualment s'enumeren a la taula 1.

Alfaproteobacteria		
Afipia sp.	*Cytophaga* sp.	*Parvibaculum* sp.
Bosea sp.	*Flavobacterium* sp.	*Phenylobacterium* sp.
Bradyrhizobium sp.	*Methylobacterium* sp.	*Rhizobium* sp.
Brevundimonas sp.	*Nordella* sp.	*Rhodopseudomonas* sp.
Caulobacter sp.	*Ochrobactrum* sp.	*Sphingomonas* sp.
Betaproteobacteria		
Acidovorax sp.	*Duganella* sp.	*Methylibium* sp.
Alcaligenes sp.	*Herminiimonas* sp.	*Polaromonas* sp.
Aquabacterium sp.	*Hydrogenophaga* sp.	*Ralstonia* sp.
Comamonas sp.	*Janthinobacterium* sp.	*Rhodoferax* sp.
Curvibacter sp.	*Leptothrix* sp.	*Variovorax* sp.
Deftia sp.	*Limnobacter* sp.	
Gammaproteobacteria		
Acinetobacter sp.	*Pseudomonas* sp.	*Stenotrophomonas* sp.
Aeromonas sp.		
Actinobacteria		
Arthrobacter sp.	*Microbacterium* sp.	*Mycobacterium* sp.
Dietzia sp.		
Firmicutes		
Bacillus sp.	*Lysinibacillus* sp.	*Staphylococcus* sp.

Taula 1. Grups majoritaris de microorganismes aïllats a les aigües minerals naturals. Elaboració pròpia a partir de: (Bischofberger et al., 1990; Casanovas-Massana and Blanch, 2012; Daood, 2008; Falcone-Dias et al., 2012; Guillot and Leclerc, 1993; Loy et al., 2005; Manaia et al., 1990; Mavridou, 1992; Otterholt and Charnock, 2011; Vachee et al., 1997).

Actualment l'aigua mineral es categoritza segons les característiques fisicoquímiques (Taula 2), no s'utilitza la microbiota autòctona com a element distintiu i característic de la qualitat de les aigües.

Classificació	Tipus d'aigua
Total de sals dissoltes	Aigua mineralització molt feble, feble, mitjana i forta
Contingut $CaCO_3$ (duresa)	Tova i dura
Composició ions Ca^{2+} i ions Mg^{2+}	Molt dolça, dolça, mitjanament dolça, mitjanament dura, dura i molt dura
En funció de l'anió i del catió principal	Bicarbonatada, sulfatada, clorurada, càlcica, magnèsica, sòdica, fluorada i ferruginosa

Taula 2. Classificació de les aigües en funció de les característiques químiques. Elaboració pròpia a partir de: (Anonymous, 2013).

1.4.2. Les comunitats microbianes de les aigües de xarxa de distribució

Els microorganismes del fílum *Proteobacteria* són els microorganismes més comuns aïllats en l'aigua durant els tractaments de potabilització i a la xarxa de distribució, aquests es llisten a la taula 3a. Només en la situació en que no s'hagi preservat la integritat del sistema, degut a una fallida en el sistema de distribució, o per la penetració d'aigua residual en el sistema, provocaria una contaminació fecal i patògens entèrics o patògens oportunistes s'introduirien en el sistema, sent un risc real per a la població amb conseqüències sanitàries importants. A la taula 3b es mostren alguns patògens oportunistes o patògens que s'han detectat a l'aigua potable de distribució després d'una contaminació (Fricker, 2003).

3a

Alfaproteobacteria		
Altererythrobacter sp.	*Erythromicrobium* sp.	*Phenylobacterium* sp.
Blastochloris sp.	*Hyphomicrobium* sp.	*Porphyrobacter* sp.
Blastomonas sp.	*Methylobacterium* sp.	*Rhodoplanes* sp.
Bosea sp.	*Methylocystis* sp.	*Ruegeria* sp.
Bradyrhizobium sp.	*Nitrobacter* sp.	*Sphingobium* sp.
Brevundimonas sp.	*Novosphingobium* sp.	*Sphingomonas* sp.
Caulobacter sp.	*Paracoccus* sp.	*Wolbachia* sp.
Betaproteobacteria		
Acidovorax sp.	*Herbaspirillum* sp.	*Paucibacter* sp.
Alcaligenes sp.	*Hydrogenophaga* sp.	*Polaromonas* sp.
Chromobacterium sp.	*Ideonella* sp.	*Ralstonia* sp.
Comamonas sp.	*Janthinobacterium* sp.	*Rhodoferax* sp.
Cupriavidus sp.	*Limnobacter* sp.	*Rubrivivax* sp.
Curvibacter sp.	*Massilia* sp.	*Schlegelella* sp.
Dechloromonas sp.	*Methylibium* sp.	*Sideroxydans* sp.
Delftia sp.	*Methylophilus* sp.	*Simonsiella* sp.
Denitratisoma sp.	*Methylotenera* sp.	*Sphaerotilus* sp.
Duganella sp.	*Methylovorus* sp.	*Thiobacillus* sp.
Ferribacterium sp.	*Nitrosomonas* sp.	*Variovorax* sp.
Gallionella sp.	*Nitrosospira* sp.	
Gammaproteobacteria		
Beggiatoa sp.	*Methylomonas* sp.	*Rheinheimera* sp.
Marinomonas sp.	*Perlucidibaca* sp.	*Rhodanobacter* sp.
Methylobacter sp.	*Pseudoxanthomonas* sp.	*Thioalkalispira* sp.
Methylocaldum sp.	*Psychrobacter* sp.	*Xanthomonas* sp.
Deltaproteobacteria	***Epsilonproteobacteria***	
Desulfovibrio sp.	*Sulfuricurvum* sp.	
Actinobacteria		
Aeromicrobium sp.	*Geodermatophilus* sp.	*Propionibacterium* sp.
Blastococcus sp.	*Gordonia* sp.	*Rathayibacter* sp.
Brevibacterium sp.	*Micrococcus* sp.	*Rhodococcus* sp.
Cellulomonas sp.	*Nocardia* sp.	
Cryobacterium sp.	*Nostocoida* sp.	
Firmicutes		
Alicyclobacillus sp.	*Brevibacillus* sp.	*Paenibacillus* sp.
Anoxybacillus sp.	*Finegoldia* sp.	
Bacteroidetes		
Arcicella sp.	*Dyadobacter* sp.	*Owenweeksia* sp.
Capnocytophaga sp.	*Flavobacterium* sp.	*Riemerella* sp.
Cytophaga sp.	*Flexibacter* sp.	*Runella* sp.

3b

Patògens oportunistes, Patògens, Indicadors		
Acinetobacter sp.	*Enterococcus* sp.	*Salmonella* sp.
Aeromonas sp.	*Escherichia coli*	*Shigella* sp.
Campylobacter sp.	*Klebsiella* sp.	*Serratia liquefaciens*
Citrobacter freundii	*Legionella* sp.	*Stenotrophomonas maltophilia*
Corynebacterium sp.	*Moraxella* sp.	*Yersinia enterocolitica*
Enterobacter sp.	*Pseudomonas* sp.	

Taula 3. Microorganismes aïllats en l'aigua durant el procés de potabilització i en l'aigua de xarxa de distribució, a: microbiota habitual, b: patògens oportunistes, patògens i indicadors. Elaboració pròpia a partir de: (Allen et al., 2004; Bharath et al., 2003; Diduch et al., 2016; Eichler et al., 2006; Kormas et al., 2010; LeChevallier et al., 1980; Navarro-Noya et al., 2013; Pinto et al., 2012; Poitelon et al., 2009; Revetta et al., 2010; Rosenberg, 2003; Tokajian et al., 2005; Vaz-Moreira et al., 2013; Zamberlan da Silva et al., 2008).

En l'aigua potable, després de la desinfecció, la concentració HPC assoleix valors de 10 UFC/ml o menors. Un dels problemes associats durant el procés de potabilització de l'aigua i en la distribució de l'aigua potable és el recreixement de les poblacions heteròtrofes. És a dir un increment en el nombre de HPC en l'aigua. De fet, els microorganismes involucrats en el recreixement són aquells presents en la pròpia aigua. El recreixement de les poblacions heteròtrofes en l'aigua de distribució depèn de molts factors: de l'eficiència dels tractaments, de la concentració del compostos orgànics i d'agents desinfectants, de la temperatura, de les condicions d'emmagatzematge de l'aigua... (Allen et al., 2004). Nivells de clor residual lliure entre 0,3 a 0,8 mg/l eviten la formació de biofilm i del recreixement de la població bacteriana. El recreixement es creu que està controlat per la quantitat de nutrients, principalment C, N i P amb una proporció 100:10:1, adequada pel creixement de les cèl·lules bacterianes (Chowdhury, 2012). Normalment, el fòsfor és l'element limitant perquè tingui lloc el recreixement (Sathasivan and Ohgaki, 1999). Diversos estudis han demostrat una correlació positiva entre el nombre de HPC i l'AOC. Estacions estivals, quan la temperatura de l'aigua és major, com també increments en la terbolesa de l'aigua, són factors de risc ja que tots els estudis presenten una correlació positiva per aquests dos paràmetres. L'alcalinització de l'aigua redueix la capacitat de desinfecció i accelera el recreixement. Tanmateix, increments en el

nombre de HPC poden produir canvis en les característiques organolèptiques de l'aigua. La taula 4 resumeix diferents factors que es correlacionen amb increments de HPC.

Estudi	CT	CR	AOC	pH	T	Terbolesa	Nitrits
(LeChevallier et al., 1987)	-0.61	-0.58		0.63	0.39	0.44	
(Wolfe et al., 1990)	-0.3 a -0.83				0.54 a 0.84		0.57 a 0.74
(Power and Nagy, 1999)	-0.37 a -0.44	-0.38 a -0.5			0.43 a 0.60	0.19 a 0.29	0.52 a 0.60
(Carter et al., 2000)		-0.43	0.32 a 0.33	0.29 a 0.53	0.20 a 0.29		
(Escobar et al., 2001)			0.85 a 0.98				

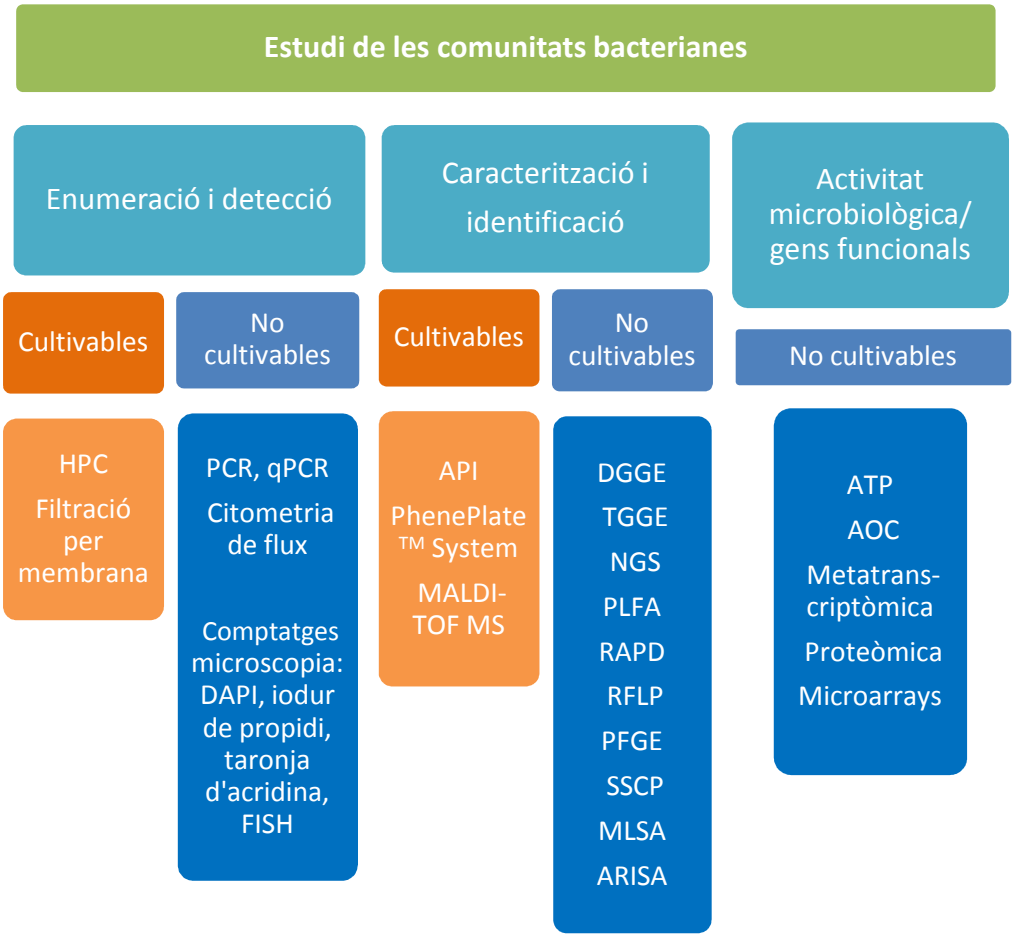
Taula 4. Correlació de HPC amb diferents factors (Pearson r), CT: clor total, CR: clor residual lliure, AOC: carboni orgànic assimilable, T: temperatura. Taula modificada parcialment (Chowdhury, 2012).

Tot i no estar regulat a la normativa, durant el procés de potabilització de l'aigua, els recomptes d'heteròtrofs podrien tenir les següents utilitats (Robertson and Brooks, 2003):

- Test de rutina per monitoritzar el funcionament dels tractaments.
- Validar i verificar l'eficàcia de nous tractaments o modificacions que es volen implantar al procés de tractament de l'aigua.

1.5. Metodologies per a l'estudi de les comunitats bacterianes de l'aigua

El desenvolupament de noves tecnologies és cabdal per a l'estudi de la diversitat microbiana. A la taula 5 s'enumeren les diferents tècniques per estudiar la comunitat bacteriana en aigües de consum, en funció si permeten la detecció i enumeració, la identificació i la caracterització o l'estudi de l'activitat i els gens funcionals.



Taula 5. Esquema de les tècniques disponibles per caracteritzar les comunitats microbianes. Taula modificada parcialment (Douterelo et al., 2014). HPC: recompte d'heteròtrofs en placa, PCR: reacció en cadena de la polimerasa, qPCR: PCR a temps real, FISH: hibridació fluorescent *in situ*, MALDI-TOF MS: espectrometria de masses de desorció/ionització mitjançant làser assistida per matriu, acoblada a un analitzador de temps de vol, DGGE: electroforesi en gel en gradient desnaturalitzant, TGGE: electroforesi en gel en gradient de temperatura, NGS: seqüenciació massiva, PLFA: anàlisi dels àcids grassos fosfolípids, RAPD: amplificació aleatòria d'ADN polimòrfic, RFLP: polimorfisme de longitud dels fragments de restricció, PFGE: electroforesi en gel de camp polsat, SSCP: polimorfisme de conformació de cadena simple, MLSA: anàlisi multilocus de les seqüències, ARISA: anàlisi automatitzat d'espaiadors ribosomals intergènics, ATP: test de detecció de trifosfat d'adenosina, AOC: mesures del carboni orgànic assimilable.

Les tècniques es poden classificar en dos grans grups: tècniques dependents de cultiu o no dependents de cultiu.

Les tècniques dependents de cultiu han estat utilitzades tradicionalment per a monitoritzar la qualitat de l'aigua. Malgrat la seva efectivitat per aquest ús, per a estudis de diversitat microbiana només una proporció molt petita de la comunitat microbiana de l'aigua pot ser recuperada ja que aproximadament <1% és cultivable (Riesenfeld et al., 2004). Tot i així, aquestes tècniques són àmpliament utilitzades en els laboratoris de rutina d'anàlisis d'aigües.

Les tècniques moleculars permeten eludir les limitacions de cultiu dels microorganismes en que es basen els mètodes dependents de cultiu. Aquestes tècniques moleculars no dependents de cultiu han permès introduir noves perspectives en l'estudi de l'ecologia microbiana i obtenir aproximacions més reals de les comunitats de l'aigua.

Tot i que cada tècnica té les seves limitacions, segons la tipologia, l'objectiu o l'aplicació de l'estudi o de l'anàlisi, moltes tècniques poden ser vàlides o la combinació d'elles poden ser complementàries. Per exemple, tècniques amb una sensibilitat més bona poden no ser factibles pel cost o per la dificultat d'anàlisi de les dades, en canvi altres tècniques dependents de cultiu poden aportar rapidesa en l'obtenció dels resultats i operativitat per les empreses d'anàlisi d'aigües.

A continuació es detallen les tècniques que s'han emprat en la present tesi doctoral per a l'estudi de les comunitats bacterianes de les aigües de consum.

1.5.1. Identificació fenotípica mitjançant espectrometria de masses de desorció / ionització amb làser assistida per matriu acoblada amb un analitzador de temps de vol

L'espectrometria de masses MALDI-TOF MS aplicada a la identificació de microorganismes, per les seves sigles en anglès *Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry* és una tècnica d'ionització suau utilitzada en espectrometria de masses que permet l'anàlisi de biomolècules. L'espectrometria de masses és una tècnica molt utilitzada en el camp de la química, però no va ser fins al 1975 que Anhalt and Fenselau (Anhalt and Fenselau, 1975) van proposar l'aplicació d'aquesta tècnica per a la identificació de microorganismes. Tot i que, no va ser fins als darrers anys de la dècada dels 80, que gràcies al desenvolupament de tècniques d'ionització suau com el MALDI va ser possible l'anàlisi de biomolècules llargues mitjançant espectrometria de masses (Cain et al., 1994; Girault et al., 1996). En aquest sentit, els primer treball publicat en relació a l'obtenció de perfils proteics sense un tractament previ de la mostra va ser a l'any 1996 (Holland et al., 1996).

MALDI-TOF MS és una tècnica ràpida, d'aplicació senzilla, acurada i de baix cost per a la identificació i la caracterització de microorganismes. MALDI-TOF MS genera un espectre de masses que és únic per a cada espècie. Aquesta signatura proteica única per a cada microorganisme permet identificar-lo. MALDI-TOF MS utilitza el càlcul del temps de vol de les proteïnes a través d'un trajecte per a confeccionar un espectre de masses, prèvia desorció/ionització làser de les molècules en un matriu determinada.

Actualment en el mercat poques empreses comercialitzen aquesta tecnologia, entre les quals destaquen: Bruker Daltonics (Alemanya), VITEK® MS-Biomérieux (França) i Schimadzu Corporation (Japó). En aquesta tesi s'ha emprat la tecnologia MALDI-TOF MS de l'empresa Bruker Daltonics per a la identificació de les soques bacterianes aïllades.

Figura 9. Imatge d'un instrument MALDI-TOF MS de Bruker Daltonics. Font: ("Bruker: High-performance scientific instruments and solutions for molecular and materials research, as well as for industrial and applied analysis," 2016)

El material biològic bacterià (quantitat visible, 10^5 cèl·lules) es mescla amb un matriu sobre una superfície conductiva metàl·lica, aquesta matriu es cristal·litza en deixar-la assecar a temperatura ambient. Les funcions de la matriu són dues: primer exposar les proteïnes intracel·lulars mitjançant la ruptura de la membrana cel·lular i a continuació facilitar la vaporització i ionització de les proteïnes. La mostra cristal·litzada és bombardejada amb el làser polsant, la matriu absorbeix l'energia del làser facilitant la desorció dels analits que són vaporitzats i ionitzats (Fig. 10). Existeixen diverses matrius, normalment s'utilitza una solució d'àcid alfa-ciano-4-hidroxicinamic (HCCA) en una barreja de solvents orgànics (acetonitril, etanol, metanol) i un àcid com l'àcid trifluoroacètic. El solvent i l'àcid faciliten la dissolució del HCCA i l'extracció de les proteïnes (Welker, 2011). Altres compostos utilitzats per a les matrius són DHB (àcid 2,5-dihydroxybenzoic), FA (àcid ferùlic), SA (àcid sinapínic) i DHAP (2,6-dihydroxyacetophenone). De fet, s'observen diferències mínimes entre els espectres de masses utilitzant diferents matrius, bàsicament aquestes diferències són en la intensitat dels pics, però no comprometen la identificació de la mostra (Croxatto et al., 2012; Welker, 2011). Per aquells microorganismes amb una paret bacteriana més rígida, com els llevats i micobacteris, que per les seves característiques no poden ser identificats per transferència directa, es necessari realitzar una extracció prèvia de les proteïnes (Bizzini et al., 2011).

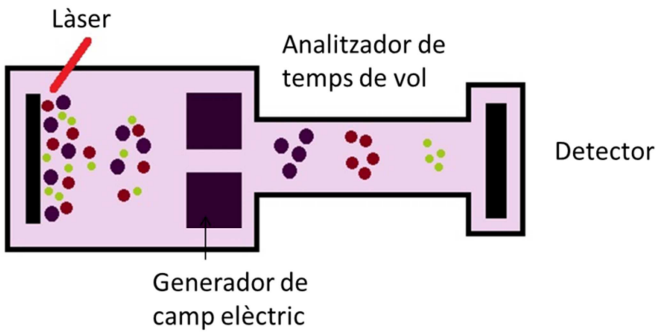


Figura 10. Esquema del principi d'operació de MALDI-TOF MS. Elaboració pròpia.

La matriu facilita la formació d'ions amb una sola càrrega, les molècules ionitzades són accelerades a través d'un camp electrostàtic i conduïdes en un tub de metall al buit fins arribar a l'extrem on es troba el detector. En funció de la relació entre la massa (m) i la càrrega (z) de cada molècula (m/z), el temps de vol variarà. D'aquesta manera, les proteïnes amb una relació m/z baixa arribaran primer al detector, i es confeccionarà l'espectre de masses caracteritzat per la relació m/z i la intensitat dels ions, aquesta dependrà de la quantitat de ions d'una determinada m/z que arribin al detector (Fig. 11).

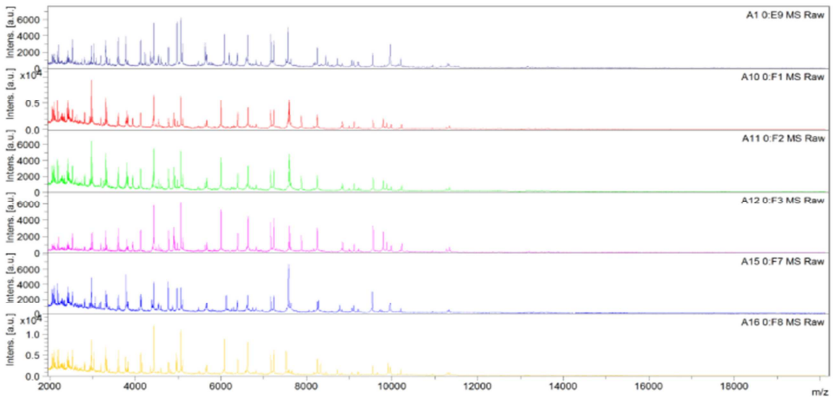


Figura 11. Espectres proteics obtinguts de soques aïllades en aigües potables.

Els perfils proteics obtinguts es comparen per similitud amb la base de dades d'espectres del fabricant de l'instrument. Els pics obtinguts es comparen amb els pics de referència d'aquelles soques dipositades a la basa de dades. De tal manera, que la similitud es representa amb un puntuació logarítmica, que comprèn valors entre 0 a 3,000. La puntuació està categoritzada en 3 subgrups en funció del nivell d'identificació, així que valors entre 2,000-3,000 indiquen una identificació d'alta confiança, a nivell d'espècie, entre 1,999-1,700 identificació a nivell de gènere i valors per sota <1,699 no és possible una identificació de la mostra.

El MALDI-TOF MS detecta les proteïnes majoritàries, així doncs, la majoria dels pics es poden assignar a proteïnes ribosomals (20% del total de les proteïnes de les cèl·lules). Els altres pics es poden associar altres proteïnes abundants com *acid-binding proteins*, ARN xaperones, *cold-shock proteins*, proteïnes de la divisió cel·lular i del metabolisme primari (Dieckmann et al., 2010, 2008; Welker and Moore, 2011). Les proteïnes ribosomals i les ARN xaperones s'expressen en gran quantitat quan la cèl·lula està en procés de divisió cel·lular. Per aquest motiu, és important que el microorganisme a identificar es trobi en la fase exponencial, d'aquesta manera el perfil proteic obtingut serà estable entre el rang de 2.000 a 20.000 m/z (Valentine et al., 2005; Wunschel et al., 2005).

El medi de cultiu no influeix en l'obtenció dels perfils proteics, ja que com s'ha mencionat anteriorment, les proteïnes que es detecten són molt abundants a la cèl·lula especialment en la fase de divisió cel·lular. Així doncs, els microorganismes cultivats amb medis diferents, l'espectre obtingut és similar (Welker and Moore, 2011).

L'estabilitat de la tècnica consisteix en que existeixi variabilitat entre espècies però no intraespècies per tal d'obtenir un patró prou sòlid per a identificar les espècies. Les diferències entre espectres de microorganismes diferents són prou evidents, a més, els

espectres de masses d'espècies properes són més semblants. És el cas de la família *Enterobacteriaceae*, que un nombre de pics es detecten en totes les espècies del grup (Lynn et al., 1999; Pribil and Fenselau, 2005). Tot i que, generalment existeix una alta similitud entre espectres de la mateixa espècie, hi ha grups que presenten una alta variabilitat intraespecífica. Per exemple, diferències intraespecífiques han estat reportades en el grup dels cocs Gram positius anaerobis (Veloo et al., 2011) com les espècies *Streptococcus pneumoniae* i *Streptococcus parasanguinis* (Vandamme et al., 1998) i amb fongs filamentosos (Seyfarth et al., 2008). No tots els grups de microorganismes presenten bones identificacions, MALDI-TOF MS no pot distingir *Shigella* spp., sinó que és identificada com *E. coli* perquè tenen un espectre molt semblant (García et al., 2012).

Aquesta tècnica té un gran potencial per ser aplicada en molts camps diferents: diagnosi clínic, seguretat alimentària i monitorització ambiental. De fet, MALDI-TOF MS ha revolucionat la identificació rutinària microbiològica en els laboratoris de microbiologia d'hospitals i centres mèdics. La majoria dels mètodes de diagnosi utilitzats requereixen el cultiu de la soca aïllada, la tecnologia MALDI-TOF MS permet obtenir la identificació en pocs minuts, en comparació amb altres tècniques fenotípiques i/o genotípiques que requereixen més de 24 hores. Molts treballs han valorat l'efectivitat i han validat aquesta tècnica per a la identificació de microorganismes patògens que causen infeccions i han contribuït a desenvolupar una base de dades d'espectres proteics robusta per facilitar la identificació dels aïllaments clínics (Alatoom et al., 2011; Barberis et al., 2014; Calderaro et al., 2014; Hsieh et al., 2008; Seng et al., 2010; Vila et al., 2012). S'ha demostrat que MALDI-TOF MS també pot distingir espècies resistents a antibiòtics, per exemple soques de *Staphylococcus aureus* resistents a meticil·lina, que difereixen de les soques susceptibles entre el rang de massa de 500-3.500 Da (Du et al., 2002). També s'ha pogut detectar soques d'*Escherichia coli* resistents a ampicil·lina, ja que es detecta un pic a 29.000 Da que correspon a una β-lactamasa. Encara és necessari aprofundir en aquests aspectes per tal de crear bases de dades suficientment robustes (Camara and Hays, 2007). Actualment aquesta tècnica ja ha estat incorporada en els protocols de rutina per a la identificació aïllaments clínics i ha desplaçat tècniques convencionals d'identificació fenotípica (Bizzini and Greub, 2010; Carbonnelle et al., 2011; Croxatto et al., 2012; Saffert et al., 2011; Seng et al., 2009).

MALDI-TOF MS ofereix potencials per ser aplicats en controls de rutina de mostres ambientals, per exemple per a la identificació d'aïllaments procedents de xarxes de

distribució d'aigua potable, estacions de tractament d'aigües, cribratge en estudis d'ecologia microbiana... Actualment la capacitat d'identificació dels microorganismes ambientals és limitada, ja que dels centenars de milions d'espècies que s'estima que existeixen a la Terra en el medi ambient (Wilson, 2003), una proporció petita estan descrites i caracteritzades. Tanmateix, només una fracció d'aquestes espècies estan incloses en les bases de dades de perfils proteics, per tant és evident la limitació per soques ambientals.

Alguns treballs han començat a explorar els potencials d'aquesta tècnica per a identificar soques aïllades de sediments hipersalins (Munoz et al., 2011), rizobacteris del sòl que poden tenir un efecte promotor del creixement de les plantes en l'altiplà central dels Andes (Ghyselinck et al., 2013), bacteris aïllats de sòls contaminats (Uhlik et al., 2011), soques d'*Aeromonas* aïllades en mostres ambientals (Benagli et al., 2012), soques aïllades de diferents tipus d'aigua d'una planta de processat d'espinacs (Hausdorf et al., 2013), bacteris d'esponges marines (Dieckmann et al., 2005) i per soques procedents d'aigua de llast (Emami et al., 2012).

1.5.2. Identificació fenotípica mitjançant API® System

Les galeries API® System comercialitzat per BioMérieux són una metodologia per a la identificació bacteriana. API® és un sistema estandarditzat de fenotipat bioquímic àmpliament utilitzat en els laboratoris d'anàlisis. Les galeries API® contenen diversos microtubs (20 o 50) amb un substrat deshidratat, que s'inoculen amb la suspensió bacteriana que reconstitueix els tests. Les reaccions metabòliques produïdes es tradueixen en viratges espontanis en el color o gràcies a un revelatge amb l'addició de reactius. En tot cas, les lectures de les reaccions són ràpides i s'han de realitzar juntament amb les taules de lectura per tal d'obtenir un perfil numèric i a través del catàleg o del web APIweb™ s'obté la identificació.

La gamma API® conté diversos tipus de galeries que es seleccionen en funció de la morfologia cel·lular del bacteri a identificar o la galeria amb 50 proves per les proves del

metabolisme per hidrats de carboni. La creació d'aquesta metodologia a l'any 1970 va revolucionar el mercat ja que implicava la realització d'una sèrie de proves bioquímiques difícils i metòdiques de preparar en un sistema miniaturitzat de ràpida utilització.

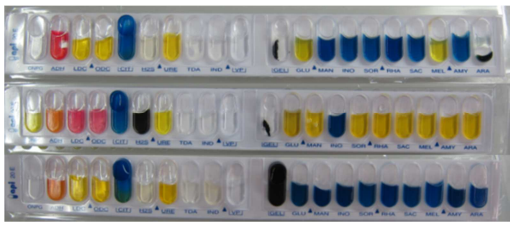


Figura 12. Imatge d'una galeria API®. Elaboració pròpia.

Aquesta metodologia ha estat utilitzada en estudis per caracteritzar soques bacterianes procedents d'aigua potable (Kühn et al., 1997; Penna et al., 2002; Ribas et al., 2000).

1.5.3. Caracterització fenotípica mitjançant Phene-Plate System™

El Phene-Plate System™ és un mètode de fenotipat bioquímic, basat en l'avaluació de la cinètica de diferents reaccions bioquímiques realitzades en un sistema miniaturitzat (comercialitzat per PhPlate Microplate Techniques AB). El Phene-Plate System™ és un mètode que permet fenotipar de forma simple un nombre elevat de soques bacterianes, així és útil per a estudis epidemiològics, nosocomials o ecològics.

Aquest fenotipat bioquímic es fonamenta en la mesura quantitativa i cinètica dels productes de reacció formats pel metabolisme bacterià a partir de diversos substrats en diferents moments. Les soques bacterianes amb fenotips idèntics, pertanyents al mateix clon, comparteixen propietats metabòliques idèntiques mentre que aïllats amb diferents fenotips tindran un comportament diferent en els processos metabòlics mesurats.

S'han desenvolupat diferents microplaques amb diferents substrats per a poder fenotipar diferents grups de microorganismes, cada prova ha estat curosament seleccionada per donar una òptima capacitat de discriminació i reproductibilitat pel grup de bacteris estudiats. Es poden diferenciar dos tipus de microplaques: plaques d'alta resolució amb 48

proves, que s'obté un fenotipat molt acurat i plaques de cribratge ràpid on cada soca es sotmet a 11 proves bioquímiques.

Les plaques PhP-48 contenen 98 pouets amb 48 tests bioquímics diferents, per tant cada placa s'utilitza per fenotipar dues soques bacterianes. Aquests tipus de microplaques són d'alta resolució i estan indicades per a qualsevol bacteri metabòlicament actiu. La taula 6 conté la relació dels substrats deshidratats de les plaques PhP-48.

Pouet	Substrat	Pouet	Substrat
1	Àcid manònic-γ-lactona	25	Sorbosa
2	L-arabinosa	26	Deoxiglucosa
3	D-xilosa	27	Deoxiribosa
4	Galactosa	28	Ramnosa
5	Maltosa	29	D-fucosa
6	Cel·lobiosa	30	L-fucosa
7	Trehalosa	31	Tagatosa
8	Palatinosa	32	Amigdalina
9	Sacarosa	33	Arbutina
10	Lactosa	34	b-metil-glucòsid
11	Melibiosa	35	5-ceto-gluconat
12	Lactulosa	36	Gluconat
13	Gentobiosa	37	Melbionat
14	Melicitosa	38	Àcid D-galacturònic
15	Rafinosa	39	Salicina
16	Inosina	40	Control de pH 5,5*
17	Adonitol	41	Citrat*
18	Inositol	42	Fumarat*
19	D-arabitol	43	Malinat*
20	Glicerol	44	Malonat*
21	Maltitol	45	Piruvat*
22	Sorbitol	46	L-tartrat*
23	Galactitol	47	Urea*
24	Control de pH 7,4	48	Ornitina*

Taula 6. Reactius deshidratats de les plaques PhP-48. * Pouets amb pH inicial àcid, i on la reacció positiva implica l'alcalinització del medi. Elaboració pròpia.

A partir dels perfils bioquímics obtinguts per a cada una de les proves i per a cada lectura, les soques s'agrupen mitjançant el mètode UPGMA (*unweighted pair group method analysis*), les soques amb un coeficient de similitud superior a 0,95 són agrupades dins d'un mateix grup fenotípic. El Phene-Plate System™ disposa d'un programa informàtic (PhPWin™) que permet fer un anàlisi de la diversitat fenotípica d'una població bacteriana amb l'índex de diversitat de Simpson, com també calcular les similituds entre les

poblacions (veure Apèndix 1). Aquest sistema de fenotipat bioquímic té la particularitat, a diferència d'altres mètodes, que per realitzar els càlculs de similitud de les soques es valora la cinètica de les reaccions produïdes a més de quantificar la utilització de cada substrat.

Aquesta metodologia és indicada per a estudis ecològics on es treballa amb un gran nombre d'aïllats i on la informació global és especialment rellevant que no la de cada soca aïllada individualment. A més, el Phene-Plate System™ és vàlid per a treballar amb microorganismes aïllats en diferents tipologies d'aigües per tal d'analitzar l'estructura de les comunitats, tal com reflecteixen diferents estudis. Així doncs, el Phene-Plate System™ ha estat utilitzat per fenotipar diferents poblacions de coliforms fecals i enterococs aïllats en aigües residuals, (Vilanova and Blanch, 2005; Vilanova et al., 2004) i per soques bacterianes en aigües de piscines naturalitzades (Casanovas-Massana and Blanch, 2013). Aquesta tècnica també s'ha emprat per comparar les poblacions heteròtrofes de diferents fonts utilitzades per envasar aigua mineral natural demostrant diferències entre les fonts (Casanovas-Massana and Blanch, 2012).

1.5.4. Caracterització genotípica mitjançant electroforesi amb gel de gradient desnaturalitzant

Electroforesi amb gel de gradient desnaturalitzant, en anglès, *Denaturing Gradient Gel Electrophoresis* (DGGE) és una tècnica genotípica que permet obtenir un perfil de la població analitzada (Muyzer et al., 1993). Aquesta tècnica consisteix en la separació per electroforesi dels amplificats d'ADN en funció de les diferències entre seqüències utilitzant gels de poliacrilamida que incorporen un gradient creixent de substàncies desnaturalitzants (urea i formamida). El gen marcador utilitzat en estudis d'ecologia microbiana és el gen que codifica per l'ARN ribosomal de la subunitat 30S dels ribosomes dels procariotes, el gen ARNr 16S. Aquest gen té regions altament conservades entre dominis i regions variables entre espècies properes. El fonament teòric de la DGGE radica en les propietats fisicoquímiques de les molècules d'ADN. Cada molècula conté dominis

amb temperatures de fusió característiques, de tal manera que, quan les molècules d'ADN assoleixen una determinada concentració d'agent químic desnaturalitzant, les molècules es desnaturalitzen. La molècula perd l'estructura de doble hèlix i pateix un canvi de conformació, fet que produeix una disminució de la mobilitat electroforètica del fragment. Per tant, cada fragment es desnaturalitzarà en una posició diferent del gel i mostrarà un patró de migració diferent, donant lloc a un patró de bandes (Muyzer et al., 1993). La posició de la banda en el gel dependrà del gradient de desnaturalització aplicat, de la duració de l'electroforesi i del camp electroforètic aplicat. Així, quan un domini presenta un elevat contingut en adenines i timines, requerirà per desnaturalitzar-se una concentració d'urea i formamida menor que una seqüència amb un major contingut de guanines i citosines.

La sensibilitat de la DGGE es pot augmentar si s'afegeix a les molècules una seqüència rica en guanines i citosines a l'extrem 5' d'un dels encebadors que serà coamplificat i introduït a la molècula d'ADN. Aquest fragment és de difícil desnaturalització i evita que les dues cadenes es separin totalment, obtenint així una major resolució. De fet, la resolució òptima s'obté quan els fragments no es desnaturalitzen per complert (Myers et al., 1985; Sheffield et al., 1989).

El perfil de bandes que s'obté és un indicatiu de la diversitat microbiana que constitueix la mostra. La DGGE és particularment útil quan es vol comparar simultàniament l'estructura de diferents comunitats. A més, les bandes es poden extreure i seqüenciar-les per tal de conèixer la seqüència de nucleòtids i poder identificar el fragment per similitud amb les bases de dades existents.

Tanmateix existeixen una sèrie de limitacions associades a la utilització de tècniques moleculars (Muyzer and Smalla, 1998; Reysenbach et al., 1992; Suzuki and Giovannoni, 1996):

- Extracció diferencial del material genètic de les espècies de la mostra que presenten diferent resistència als tractaments de lisis.
- Amplificacions esbiaixades a la reacció de PCR (reacció en cadena de la polimerasa): menor amplificació de seqüències amb un elevat contingut de GCs i variació del nombre de còpies del gen en diferents espècies.
- Formació de quimeres i heterodúplexs.

O limitacions intrínseques de la pròpia tècnica (Muyzer and Smalla, 1998; Myers et al., 1985):

- Detecció de fragments d'ADN de fins a 500pb.
- El gen ARNr 16S que presenta varies còpies en un mateix organisme, es poden obtenir diferents bandes per una mateixa espècie. El nombre de còpies de l'operó ribosòmic varia entre 1 i 15 segons l'espècie, el gènere o la família.
- Teòricament, amplificats d'ADN amb diferent composició nucleica es desnaturalitzaran en diferents posicions del gel, però si no s'ha optimitzat prou bé el gradient, es pot obtenir bandes úniques que representin diferents microorganismes.
- Detecció d'aquells microorganismes que representin més d'un 3% de la població, ja que per sota d'aquest valor no poden ser detectats.

Tot i les limitacions, la DGGE reflecteix de manera aproximada la composició de la microbiota d'una comunitat. Diferents estudis han utilitzat aquesta metodologia per a l'estudi de les comunitats d'aigües minerals i d'aigües de distribució. Aquí es s'enumeren una sèrie d'estudis que han aplicat aquesta tècnica: estudis per caracteritzar la comunitat microbiana de diferents d'aigües minerals i variacions d'aquestes amb el temps (Burtscher et al., 2009; Dewettinck et al., 2001; França et al., 2014), estudis per a avaluar la presència de patògens oportunistes en biofilms en aigües de distribució (Pryor et al., 2004) o per a monitoritzar la formació de biofilm en aquests sistemes (Boe-Hansen et al., 2003). També per a avaluar la influència dels tractaments d'aigua sobre la comunitat bacteriana (Vaz-Moreira et al., 2013) o per a avaluar la qualitat de l'aigua de distribució (Sekar et al., 2012).

1.5.5. Enumeració de cèl·lules mitjançant Live/dead BacLight™

Live/Dead BacLight™ és un test de fluorescència per a l'enumeració de cèl·lules i avaluar la viabilitat d'aquestes. Aquest test conté dos fluorocroms intercalants d'àcids nucleics: SYTO®9 (colorant d'àcid nucleic verd fluorescent) i iodur de propidi (colorant d'àcid nucleic vermell fluorescent). Aquests fluorocroms difereixen en la capacitat de penetrar a l'interior de les cèl·lules i en l'espectre d'emissió de fluorescència. El colorant SYTO®9 penetra a totes les cèl·lules, en canvi, el iodur de propidi només en aquelles que tenen la membrana malmesa, és a dir, no intacta. El iodur de propidi causa una reducció del SYTO®9 en presència dels dos fluorocroms. De tal manera que les cèl·lules amb la membrana intacta es visualitzaran verdes al microscopi de fluorescència mentre que les cèl·lules danyades de color vermell. L'excitació/emissió màxima per aquests fluorocroms són 480/500 nm pel SYTO®9 i 490/635 nm pel iodur de propidi.

Live/Dead BacLight™ permet quantificar i distingir les cèl·lules viables i no viables en pocs minuts, inclús si la població és diversa. Alguns estudis han aplicat aquest test pel recompte de cèl·lules de l'aigua mineral (Defives et al., 1999; Ramalho et al., 2001).

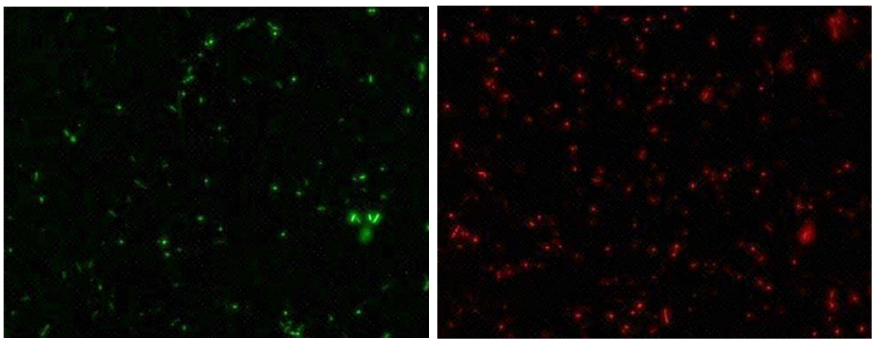


Figura 13. Imatge de microscòpia de fluorescència a 1.000 augments, cèl·lules viables en verd (dreta) i cèl·lules no viables en vermell (esquerra) d'un aigua mineral natural.

1.6. Riscos microbiològics associats a l'aigua de consum

1.6.1. Contaminació fecal

Fins aquest darrer any, no s'havia reportat cap cas d'infecció relacionada amb una aigua envasada que complís els criteris normatius a la Unió Europa. El passat mes d'abril del 2016 a Catalunya es declarava un brot de gastroenteritis, que va afectar a 4.136 persones de diferents municipis de les demarcacions de Tarragona i Barcelona. Les analítiques de l'aigua de deu envasada a Andorra van determinar la presència de nivells alts de norovirus.

Alguns estudis han reportat certes marques d'ampolles d'aigua envasada comercialitzades que no complien els criteris microbiològics recollits a la legislació dels seus respectius països, suposant un risc per a la salut pública. Totes elles contenien microorganismes indicadors de la contaminació fecal o patògens oportunistes. A la taula 7 es descriuen alguns d'aquests casos, tots ells són d'aigua envasada comercialitzada en països fora de la Unió Europea.

Nombre ampolles	Indicadors microbians (%)				Patògens oportunistes (%)				País	Referència
	CT	CF	EC	EF	AH	PA	SA	GL		
653	7,7	7,7			ab				Canadà	(Warburton, 1993)
136		ab		ab	1,5	3			Taiwan	(Tsai and Yu, 1997)
80						6,25			Austràlia	(Jayasekara et al., 1998)
344	5,2		5			7,6			República Trinitat i Tobago	(Bharath et al., 2003)
56	29	ab	ab			3,6	5,2	2,4	Egipte	(Abd El-Salam et al., 2008)
246	23		7	2		6			Hongria	(Varga, 2011)
324	ab	ab	ab	1,2		0,6			Brasil	(Falcone-Dias and Farache Filho, 2013)

Taula 7. Qualitat microbiològica de diferents aigües minerals naturals envasades, percentatge de mostres positives pels diferents microorganismes. CT: coliforms totals, CF: coliforms fecals, EC: *Escherichia coli*, EF: *Enterococcus faecalis*, AH: *Aeromonas hydrophila*, PA: *Pseudomonas aeruginosa*, SA: *Staphylococcus aureus*, GL: Cists de *Giardia lamblia,* ab: absència. Elaboració pròpia.

Els estudis anteriors ressalten la necessitat d'implantar un sistema de vigilància durant el procés de l'envasat, ja que algunes de les ampolles possiblement es van contaminar durant aquest procés i incideixen en l'establiment d'un procés sistemàtic preventiu per a garantir la seguretat microbiològica, l'anàlisi de perills i punts de control crítics (APPCC).

Els brots de transmissió hídrica a través de l'aigua de la xarxa de distribució tenen un gran impacte sobre la salut pel gran nombre de població exposada. Els agents patògens identificats amb major freqüència a Espanya en els darrers 20 anys que han causat brots han estat *Campylobacter jejuni* i *Salmonella typhimurium.* Per altra banda, han incrementat els brots de tipologia vírica, com norovirus, probablement per la major resistència que ofereixen als tractaments químics. Tot i així, els brots de transmissió hídrica són poc freqüents en els països més desenvolupats, sobretot a les ciutats, algun cas es produeix més freqüentment a les zones rurals i generalment en el període estival. Contràriament, els brots d'infecció hídrica són habituals en els països econòmicament més desfavorits (Jofre, 2010).

Esporàdicament, algun brot associat a l'aigua de distribució ha tingut lloc en els països més desenvolupats. Per exemple, a Montrose (Escòcia) un problema en el sistema de cloració de l'aigua de distribució va produir un brot causat per *Shigella sonnei* (Green et al., 1968). A Fife (Estats Units), l'aigua residual va contaminar l'aigua de distribució amb *E. coli* O157 i *Campylobacter* produint una infecció a la població (Jones and Roworth, 1996). A la ciutat de Nokia (Finlàndia), al 2007, l'aigua residual es va mesclar amb l'aigua de xarxa, com a conseqüència el 42% de la població va tenir algun símptoma d'infecció, els patògens que es van aïllar dels pacients amb més freqüència van ser norovirus i *Campylobacter jejuni* (Räsänen et al., 2010). També es va produir un brot en una petita localitat de Catalunya, l'any 2006, a València d'Àneu (Lleida) va tenir lloc un brot de gastroenteritis pel consum d'aigua de xarxa contaminada per *Shigella sonnei* que va afectar un 65% de la població. Un abocament d'aigua residual il·legal a prop del punt de captació de l'aigua de subministrament en va ser l'origen i no vas ser corregit perquè el clorador municipal no estava en funcionament en aquell moment (Godoy et al., 2011).

Les plantes de tractament d'aigua poden no fer front a un increment del nombre de patògens de l'aigua a tractar degut a les pluges torrencials que remouen els sediments del riu i els patògens retinguts als sediments queden en suspensió a l'aigua incrementant així la càrrega bacteriana (Curriero et al., 2001). Per exemple, l'any 1993 a Milwaukee (Estats Units), es va produir un brot de criptosporidiosi degut a un increment dels nivells de

Cryptosporidium de l'aigua després d'una precipitació intensa i que la planta de tractament no va poder eliminar (Mac Kenzie et al., 1994). Per exemple, una situació semblant va tenir lloc a Catalunya, un brot pel consum d'aigua de subministrament contaminada per *Shigella sonnei* va afectar a la població de Santa Maria de Palautordera, l'any 2002. Una pluja intensa va remoure els fangs, deteriorant així la qualitat de l'aigua de captació. La planta de tractament d'aigua no estava preparada per tractar aigua amb una terbolesa més alta, a més el personal de planta no va saber gestionar la situació d'excepcionalitat adequadament i l'aigua va ser clorada amb uns nivells més baixos dels que es requerien en aquella situació (Arias et al., 2006).

1.6.2. Resistència a antibiòtics

La resistència a antibiòtics és una propietat intrínseca que poden tenir certs bacteris (Andersson and Hughes, 2011; Cantón and Morosini, 2011). De fet, en el medi es detecten resistències a antibiòtics en ambients amb pocs impactes antropogènics (Allen et al., 2010; Segawa et al., 2013). Alguns estudis han valorat la presència de microorganismes resistents a antibiòtics en aigua mineral (Falcone-Dias et al., 2012; Mary et al., 2000; Massa et al., 1995; Messi et al., 2005). Així doncs, en aigües minerals comercialitzades en diferents països s'han aïllat nombroses soques pertanyents a diferents gèneres amb resistències a antibiòtics: *Acidovorax, Afipia, Bosea, Bradyrhizobium, Brevundimonas, Ralstonia, Variovorax, Moraxella, Nordella, Pseudomonas, Flavobacterium, Pedobacter* i *Rhizobium.* Les soques presentaven resistències principalment a cloramfenicol, ampicil·lina, colistina i sulfametizol (Messi et al., 2005). En un altre estudi en que es comparaven soques de diferents orígens, cada soca aïllada presentava resistència al menys a 3 tipus d'antibiòtics. Les resistències més freqüents van ser als antibiòtics del grup dels beta-lactàmics com ticarcil·lina, ceftazidima i cefalotina i també a levofloxacin i ciprofloxacina pertanyents al grup de les quinolones (Falcone-Dias et al., 2012).

En aigua potabilitzada, malgrat la poca informació respecte al tema, s'han detectat que els gèneres *Acinetobacter, Pseudomonas, Sphingobium* i *Sphingomonas* entre altres heteròtrofs aïllats però no identificats són resistents a diferents antibiòtics com amoxicil·lina, piperacil·lina, gentamicina, meropenem, rifampicina i ticarcil·lina (Narciso-da-Rocha et al., 2013; Vaz-Moreira et al., 2011; Xi et al., 2009). En aquestes espècies la resistència probablement és intrínseca, el patró de resistència és semblant entre espècies i no difereix segons els orígens dels aïllats, tot i així es desconeix l'impacte que pot tenir sobre la salut la presència d'aquests microorganismes (Vaz-Moreira et al., 2014).

Les aigües de consum poden contenir bacteris resistents a antibiòtics, tot i així, els fenotips de resistència són possiblement intrínsecs de les espècies, per tant la probabilitat de transferència d'aquests gens a la microbiota intestinal és poc probable (Vaz-Moreira et al., 2014). Malgrat no ser gens de resistència adquirits, no s'ha de menysprear i requereixen més atenció per tal d'estudiar les implicacions que podrien suposar.

2.OBJECTIUS

2.OBJECTIUS

En la present tesi doctoral es pretén estudiar les comunitats bacterianes heteròtrofes associades a dos tipus d'aigües pel consum humà de proveïments diferents: aigües minerals envasades i aigües d'abastament. Aquestes dues tipologies d'aigües de consum fa que els objectius plantejats per cadascuna foren:

Objectius per l'estudi de la microbiota pròpia en les aigües minerals naturals envasades:

1. Avaluar els canvis en les comunitats bacterianes heteròtrofes cultivables de l'aigua mineral natural embotellada durant la seva vida comercial.

2. Aïllar i caracteritzar fenotípicament les comunitats heteròtrofes de l'aigua mineral natural.

3. Caracteritzar les comunitats bacterianes en aigües minerals embotellades mitjançant tècniques moleculars per tal d'analitzar la seva utilitat en la traçabilitat de diferents marques comercials.

Els objectius 1 i 2 han permès disposar del contingut de l'article 1, i l'objectiu 3 ho ha permès per a l'article 2.

Objectius per l'estudi de la microbiota de l'aigua d'una xarxa d'abastament:

4. Adaptar i avaluar si la tecnologia MALDI-TOF MS és útil per monitoritzar i identificar les poblacions heteròtrofes d'una estació de tractament d'aigua potable (ETAP).

5. Identificar les comunitats bacterianes heteròtrofes dominants en les diferents fases de potabilització en l'ETAP estudiada, i avaluar els canvis estacionals de les poblacions.

6. Valorar la presència de *Pseudomonas* spp. associades a l'aigua abans i després del tractament per osmosi inversa en l'ETAP.

El treball experimental realitzat per aconseguir l'objectiu 4 constitueix el contingut de l'article 3. L'objectiu 5 ha permès disposar del contingut per a la preparació de l'article 4, mentre que l'objectiu 6 de l'article 5.

3.INFORMES

3.INFORMES

3.1. Informe sobre el factor d'impacte

Els articles que formen part de la Memòria de la Tesi Doctoral presentada per Laura Sala i Comorera han estat publicats o sotmesos per a la seva publicació en revistes internacionals indexades al Journal Citation Reports® tal i com es detalla a continuació:

L'article **"Temporal variations in aerobic heterotrophic bacterial populations in bottled mineral water"** està actualment sotmès a la revista *PlosOne*. Aquesta revista es troba inclosa en el primer quartil de l'àrea de ciències multidisciplinàries amb un factor d'impacte per a l'any 2015 de 3,54.

L'article **"Evaluation of the bacterial community to assess the traceability of bottled water of different mineral water brands during the shelf life"** està actualment en fase final de revisió per a ser sotmès a *Environmental Microbiology,* que es troba inclosa en el primer quartil de l'àrea de microbiologia. L'any 2015, la revista va presentar un índex d'impacte de 5,93.

L'article **"Use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry for bacterial monitoring in routine analysis at a drinking water treatment plant"** està en impressió. L'article va ser acceptat el 11 de gener del 2016 (DOI:10.1016/j.ijheh.2016.01.001) a la revista *International Journal of Hygiene and Environmental Health,* que està dins del primer quartil de la temàtica de ciències ambientals i salut pública. L'any 2015, la revista va presentar un índex d'impacte de 3,98.

L'article **"Heterotrophic monitoring at a drinking water treatment plant by matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometry after different drinking water treatments"** està actualment sotmès a la revista *Journal of Water and Health*, que està dins del quart quartil de la temàtica de ciències ambientals i microbiologia. L'any 2015, la revista va presentar un índex d'impacte de 1,03.

L'article **"*Pseudomonas*-related populations associated with reverse osmosis in drinking water treatment"** està publicat a la revista *Journal of Environmental Management* (DOI:10.1016/j.jenvman.2016.07.089). Aquesta revista es troba inclosa en el primer quartil de l'àrea temàtica de ciències ambientals amb un factor d'impacte per a l'any 2015 de 3,13.

Dr. Anicet R. Blanch i Gisbert

Dra. Cristina García i Aljaro

Barcelona, 20 de setembre de 2016

3.2. Informe de participació de la doctoranda

La doctoranda Laura Sala i Comorera ha participat en els articles que formen part de la seva Tesi Doctoral de la manera que es detalla a continuació:

Laura Sala-Comorera, Arnau Casanovas-Massana, Cristina García-Aljaro i Anicet R. Blanch. Temporal variations in aerobic heterotrophic bacterial populations in bottled mineral water (sotmès a *PlosOne*).

La doctoranda va dur a terme les tasques experimentals, incloent l'aïllament de les soques, enumeració de les comunitats bacterianes, caracterització fenotípica i genotípica de les soques i els càlculs estadístics. D'altra banda, va participar activament en la redacció del manuscrit i en l'elaboració de les taules i figures.

Laura Sala-Comorera, Arnau Casanovas-Massana, Cristina García-Aljaro i Anicet R. Blanch. Evaluation of the bacterial community to assess the traceability of bottled water of different mineral water brands during the shelf life (en preparació).

La doctoranda va dur a terme les tasques experimentals, enumeració de les comunitats bacterianes totals i les comunitats bacterianes cultivables. Així com les anàlisis moleculars (extracció ADN i anàlisis DGGE). La doctoranda ha processat les dades i ha participat en l'elecció de les bandes de la DGGE significatives. Finalment, va clonar i seqüenciar les bandes. La doctoranda va redactar el manuscrit.

Laura Sala-Comorera, Carles Vilaró, Belén Galofré, Anicet R. Blanch i Cristina García-Aljaro. 2016. Use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry for bacterial monitoring in routine analysis at a drinking water treatment plant. *International Journal of Hygiene and Environmental Health*. Article acceptat el 11 gener de 2016, actualment en impressió, DOI:10.1016/j.ijheh.2016.01.001.

La doctoranda va dur a terme la major part de les tasques experimentals, incloent la caracterització fenotípica i genotípica de les soques aïllades, la identificació mitjançant MALDI-TOF MS i API® i els càlculs estadístics. A més a més, va participar activament en l'elaboració de les taules i figures i en la redacció de l'article.

Laura Sala-Comorera, Anicet R. Blanch, Carles Vilaró, Belén Galofré i Cristina García-Aljaro. Heterotrophic monitoring at a drinking water treatment plant by matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometry after different drinking water treatments (sotmès a *Journal of Water and Health*).

La doctoranda va dur a terme la major part de les tasques experimentals, incloent el processament de les mostres, la caracterització fenotípica i genotípica de les soques aïllades, la identificació mitjançant MALDI-TOF MS i els càlculs estadístics de diversitat poblacional i similitud poblacional. D'altra banda, va participar activament en la redacció del manuscrit i en l'elaboració de les taules i figures.

Laura Sala-Comorera, Anicet R. Blanch, Carles Vilaró, Belén Galofré i Cristina García-Aljaro. 2016. *Pseudomonas*-related populations associated with reverse osmosis in drinking water treatment. *Journal of Environmental Management,* DOI:182 (2016) 335-341. 10.1016/j.jenvman.2016.07.089.

La doctoranda va dur a terme la major part de les tasques experimentals, incloent caracterització fenotípica i genotípica de les soques aïllades, la identificació mitjançant MALDI-TOF MS, el test de susceptibilitat antimicrobiana i el test de formació de biofilm *in vitro*. Finalment, la doctoranda va elaborar les taules i les figures i va redactar l'article.

Cap dels coautors dels articles ha utilitzat les dades descrites en aquestes publicacions per a l'elaboració de la seva tesi doctoral.

Dr. Anicet R. Blanch i Gisbert Dra. Cristina García i Aljaro

Barcelona, 20 de setembre de 2016

4.PUBLICACIONS

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4.1. Article 1

Temporal variations in aerobic heterotrophic bacterial populations in bottled mineral water

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Revista: Article sotmès a PlosOne, en revisió.

La demanda d'aigua embotellada va experimentar un important creixement en els últims anys, i en aquest sentit, es preveu una tendència similar en els propers anys. L'augment de la demanda de l'aigua mineral natural envasada s'explica per diferents factors entre els quals es pot destacar la insatisfacció per les propietats organolèptiques de l'aigua de la xarxa de distribució i les campanyes comercials sobre els efectes beneficiosos per a la salut. Les fonts i els aqüífers tenen una microbiota pròpia que els caracteritza i diferencia. Segons la Directiva Europea 2009/54/CE, durant el procés d'embotellament de les aigües minerals naturals està prohibit efectuar qualsevol tractament de desinfecció que modifiquin aquesta microbiota. De fet, la concentració de la comunitat bacteriana heteròtrofa és baixa en el punt d'origen, 10 UFC/ml, però després de l'envasat, la concentració augmenta entre la primera i segona setmana i pot assolir valors de 10^4-10^5 UFC/ml.

L'objectiu d'aquest estudi va ser caracteritzar i analitzar les variacions i canvis de les comunitats heteròtrofes cultivables de l'aigua mineral natural envasada fins un any després de l'envasat.

Dues aigües minerals naturals envasades en ampolles de vidre procedents de dos punts geogràficament distanciats de l'estat espanyol es van seleccionar per tal de realitzar el seguiment de les comunitats heteròtrofes durant la seva vida comercial. Les ampolles d'aigua van ser analitzades als 55, 125 i 365 dies després de l'envasat. Aquest estudi es va realitzar per duplicat en dos lots comercials independents envasats en anys consecutius. Per a cada temps, es van aïllar 50 soques per sembra per extensió en el medi R2A a 20 ˚C, i van ser fenotipades bioquímicament mitjançant el sistema PhenePlate™ (PhPlate Microplate Techniques AB). També es va calcular l'índex de la diversitat de Simpson i la

similitud poblacional entre les comunitats. Finalment, es van identificar les soques representatives dels diferents agrupaments bioquímics mitjançant la seqüenciació del gen del ARNr 16S.

Els recomptes d'heteròtrofs en placa (HPC) no van ser constants durant l'envelliment de l'aigua dins l'ampolla, sinó que van fluctuar, les concentracions HPC es van situar a nivells de 10^3 UFC/ml. Un any després de l'envasat, encara es van detectar heteròtrofs en placa, tot i que la concentració era menor que la detectada als 55 dies. Un total de 598 soques aïllades van ser fenotipades bioquímicament. La diversitat de Simpson va resultar ser moderada, amb oscil·lacions entre els temps estudiats però amb una disminució progressiva durant l'envelliment de l'aigua. Els valors de similitud poblacional van ser inferiors a 0,2 entre la mateixa aigua en els diferents temps, per tant existien diferències significatives entre elles, i també amb els duplicats corresponents analitzats al següent any. Es va observar una successió i evolució de les poblacions microbianes cultivables dominants al llarg del temps, fet que indica la complexitat d'aquests ecosistemes. La majoria de les soques aïllades (77%) pertanyien a la classe *Alfaproteobacteria*, les soques restants es van dividir en *Gammaproteobacteria* (20%) i *Actinobacteria* (3%). El conjunt de soques aïllades pertanyien a 14 gèneres diferents. Només un gènere per cada aigua va ser identificat en la majoria dels temps estudiats i lots de la mateixa aigua, aquests gèneres van ser *Brevundimonas* i *Pseudomonas,* a l'aigua A i B respectivament.

La comunitat bacteriana heteròtrofa no és estable durant la comercialització de l'aigua, sinó que presenta fluctuacions en les comunitats. En definitiva, no es pot establir una tendència de la comunitat heteròtrofa durant l'envelliment de l'aigua. No obstant, s'observa una disminució de la diversitat i dels recomptes d'heteròtrofs després d'un any de l'envasat. La comunitat bacteriana és diferent per cada marca d'aigua mineral natural i evoluciona durant la seva vida comercial, fet que comporta canvis en els gèneres dominants. En conseqüència, es fa palès que les aigües minerals naturals envasades són microsistemes complexos.

Temporal variations in aerobic heterotrophic bacterial populations in bottled mineral water

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Abstract

Natural mineral waters contain a wide phenotypic and genetic microbial diversity. Identification of these microbial communities and their temporal variations is of great importance for consumer health and company marketing strategies. Our aim in this study was to characterise and analyse variation in cultivable aerobic heterotrophic bacteria in bottled natural mineral water over a one-year period. The experiments were performed in two successive years using two independent batches of bottled mineral water. For this purpose, two natural mineral waters bottled in glass were examined at 55, 125 and 365 days after bottling. The heterotrophic bacteria were biochemically phenotyped using the PhenePlate™ system (PhPlate Microplate Techniques AB, Sweden), and population diversity and similarity indexes among subpopulations were calculated. Representative isolates of each biochemical cluster were identified by sequencing the 16S rRNA gene. One year after bottling, heterotrophic bacteria were still present; however, we did not observe a generalisable trend that explained the behaviour of the bacterial heterotrophic community in mineral water inside the bottle. In addition, despite fluctuations in diversity, there was an overall decrease after one year in the bottle. Differences in dominant bacterial species between batches were found during shelf-life and between batches, indicating the complexity of these ecosystems.

Practical Applications

Mineral water market has become a global business. The knowledge of the microbial heterotrophic populations and how these populations change during its marketing could be added value for the companies so as to lead the market and prevent health associated risks. This work contributes towards a better understanding of the microbial communities in mineral water during storage.

Introduction

In the past 15 years, consumption of bottled natural mineral water has increased steadily worldwide (Rani and others 2012; Storey 2010), and global demand is predicted to continue rising in the near future (Rodwan 2014). This increase in bottled water consumption is due to multiple factors, namely a displeasure with the organoleptic properties of tap water and concerns about its safety (Abrahams and others 2000; Falcone-Dias and Farache Filho 2013), recognition of the beneficial and nutritional properties of natural mineral water (Varga 2011; Venieri and others 2006) and the successfully promoted perception that natural mineral water contributes to a healthier lifestyle (Bharath and others 2003; Misund and others 1999; Rodwan 2014; Varga 2011). Within the European Union, natural mineral water is defined by Directive 2009/54/EC as microbiologically wholesome water, originating in an underground water table or deposit and emerging from a spring tapped at one or more natural or bore exits (Anonymous 2009). Natural mineral water cannot be subjected to any kind of disinfection treatment that could alter its natural bacterial community, and the only authorised treatment is removal of unstable constituents and the elimination, introduction or reintroduction of carbon dioxide. Regarding its microbiological safety, within 12 hours following bottling the total aerobic heterotrophic colony count at source cannot exceed 100 CFU/ml at 20 to 22 ℃ in 72 hours and 20 CFU/ml at 37 ℃ in 24 hours. In addition, throughout its shelf-life, natural mineral water should be free from *Pseudomonas aeruginosa*, *Escherichia coli* and other coliforms and faecal streptococci in any 250 ml of sample, sporulated sulphite-reducing anaerobes in 50 ml and all parasites or pathogenic microorganisms.

Despite these strict requirements for faecal and pathogenic microorganisms, natural mineral waters are not sterile environments and contain a wide phenotypic and genetic microbial diversity (Casanovas-Massana and Blanch 2012; Falcone-Dias and Farache Filho 2013; Leclerc and Moreau 2002; Rosenberg 2003). At source, the concentration of aerobic heterotrophic bacteria is low, usually around 10 CFU/ml (Bischofberger and others 1990; França and others 2014). However, after bottling, the concentrations tend to increase, reaching up to 10^4-10^5 CFU/ml within 7-15 days (Falcone-Dias and Farache Filho 2013; Leclerc and Moreau 2002; Urmeneta and others 2000). The reasons for this bottle effect phenomenon are not well known, but it has been hypothesised that an increase in temperature, exposure to direct sunlight and bottle materials may stimulate the growth of certain bacterial populations. Once this peak is reached, the concentrations generally remain constant or decrease slightly (Bischofberger and others 1990; Sefcova 1997).

Several studies have characterised aerobic heterotrophic populations in spring water or natural mineral water immediately after bottling. However, little is known about the temporal variation

in these populations during the shelf-life of bottled water. Identification of these microbial populations and their dynamics is of great importance since changes in the concentrations and composition in the bottle may pose health risks for consumers. In addition, characterisation of aerobic heterotrophic populations could potentially be used as a tool to track the water on the market, for example to distinguish mineral water brands since different water sources may have a specific microbial signature (Casanovas-Massana and Blanch 2012). Our aim in this study was to characterise and analyse variation in cultivable aerobic heterotrophic bacteria in bottled natural mineral water over a one-year period. Although only a small portion of bacteria in mineral natural water is viable and cultivable (Allen and others 2004; França and others 2014; Leclerc and Moreau 2002), we focused on analysing the cultivable fraction, since the heterotrophic plate count is the most widely used criterion in routine microbiological water quality assessment in bottling plants (Diduch and others 2016). To this end, we selected two different natural mineral water brands, and phenotypically and genotypically characterised their heterotrophic bacteria during shelf-life. Moreover, so as to examine reproducibility between batches, another batch from a consecutive year bottled in the same period was also evaluated.

Materials and methods

Natural mineral water sampling

Two non-carbonated natural mineral water brands (A and B) from two geographically independent Spanish springs were selected for this study. One-litre glass bottles of each natural mineral water were purchased from a local retailer one month after bottling according to the packaging date. Glass bottles were stored under dark conditions at 20 ± 2 ℃ prior to analysis. To assess changes in the microbial populations over time, each brand was analysed at 55, 125 and 365 days after bottling. All the experiments were performed in two successive years using two independent batches (1 and 2) bottled in the same season from each water brand.

Enumeration and isolation of aerobic heterotrophic bacteria

All bottles were shaken vigorously before sampling to retrieve any microorganisms potentially attached to the bottle surface. For aerobic heterotrophic enumeration and to obtain isolates for further phenotypic analysis, 100 µl aliquots of each water sample were spread onto R2A plates (Pronadisa, Madrid, Spain) and incubated at 20 ± 2 ℃ for 7 days. After incubation, viable counts were enumerated and 50 colonies from each brand and each point were randomly selected and isolated by subculture on R2A plates. The number of strains isolated was assumed to be representative of the natural heterotrophic bacterial populations according to previous studies

(Bianchi and Bianchi 1982). The concentrations of aerobic heterotrophic bacteria for each brand on each day of analysis and for each batch were compared using one-way ANOVA corrected with Tukey's post-hoc test. Statistical analyses were performed using GraphPad Prism 6.03 (GraphPad Software, Inc, La Jolla, U.S.A).

Biochemical characterisation

All isolates were phenotypically characterised using PhP-48 microplates from the PhenePlate™ system (PhPlate Microplate Techniques AB, Stockholm, Sweden) following the manufacturer's instructions. PhP-48 plates are based on 96 biochemical tests with dehydrated reagents chosen to provide a high level of discrimination among species. Each plate allows the biochemical fingerprinting of two distinct isolates (48 biochemical tests per isolate). The biochemical fingerprinting procedure with these microplates has been described elsewhere (Kühn 1985). Briefly, cultures were prepared on R2A at 20 ± 2℃ for 96 ± 2 h. Cell suspensions were prepared by harvesting these cultures in a solution of distilled water at 0.1% w/v proteose peptone and 0.011% w/v bromothymol blue. After 30 minutes of agitation at 20 ± 2℃, bacterial suspension aliquots of 150 µl were inoculated into each well. The PhP-48 microplates were incubated at 20 ± 2℃ in a humid chamber to prevent dehydration. Growth was monitored at 620 nm with the iEMS Reader MF (Labsystems, Lima, Peru) at 16, 40, 64, 84 and 112 h. *Pseudomonas aeruginosa* NCTC 10332^{T} was used as an internal control to confirm reproducibility among experiments.

Phenotypic characterisation and population diversity and similarity indexes

The biochemical fingerprinting of each isolate was calculated as an average value of the five measured absorbances. All biochemical fingerprinting results were compared pairwise, yielding a correlation coefficient. A similarity matrix was obtained from all the correlation coefficients and a dendrogram was constructed using the unweighted-pair group method analysis (UPGMA) and average linkage. All isolates with a correlation coefficient higher than 0.95 were grouped together to constitute a phenotypical cluster. Isolates with the highest minimum and the highest mean similarity to all isolates within the same cluster were selected as the representative strain for further characterisation (Kühn and others 1991). The population diversity for each time-point and water brand was calculated using Simpson's diversity index (Hunter and Gaston 1988), which is a relative measure of the distribution of isolates into different types. A low value (minimum 0) indicates that one of a small number of phenotypic profiles dominates the population, whereas a high value (maximum 1) indicates a distribution of isolates into many profiles. The similarity between populations and subpopulations was calculated using the coefficient of population similarity (Sp) (Kühn and others 1991). Bacterial populations were considered not significantly different when the Sp value was greater than 0.2 (Kühn and others

1991). Clustering analysis and calculations of population diversity and similarity were performed using the PhPWin® software (PhPlate Microplate Techniques AB, Stockholm, Sweden).

Genotypic characterisation

The representative strain from those phenotypic clusters containing 3 or more isolates was selected for further genotypic characterisation. Isolates were cultured in Tryptic Soy Broth at 20 ± 2ºC for 24 ± 2 h, and DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, U.S.A.) following the manufacturer's instructions. Partial 16S rRNA gene amplification and sequencing was performed using universal primers 27f 5'(AGAGTTTGATCMTGGCTCAG)3' and 1492r 5'(TACGGYTACCTTGTTACGACTT)3' (Weisburg and others 1991). Sequences were submitted for similarity searches to the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Blast/). Sequences have been deposited in GenBank under the accession numbers shown in Table S1.

Results and discussion

Enumeration of aerobic heterotrophic bacteria

The heterotrophic colony plate count (HPC) pattern over time was different for each water brand and batch (Table 1). The HPC in the first batch of water A (A1) showed a slight but not significant increase from 55 to 125 days followed by a considerable decrease ($p<0.0001$) down to 1.99 ± 0.17 $\log_{10}$ CFU/ ml after one year. In contrast, the second batch (A2), showed a significant decrease from 55 to 125 days, but the concentration stabilised thereafter ($p<0.0001$). For brand B, the first batch (B1), which had the lowest concentration at day 55, showed an increase after 125 days and a subsequent decrease at 365 days ($p<0.0001$). However, the second batch (B2) presented a steady decrease throughout the study from 55 to 365 days ($p<0.0001$). Overall, we observed considerable fluctuations in the HPC throughout the study, although the concentration in all waters after one year of storage was always lower compared with the initial test. These results suggest that each water batch may have a different behaviour, which is in agreement with a previous study where different heterotrophic counts were found among bottles from the same batch on the same day of analysis (Falcone-Dias and Farache Filho 2013).

Several studies have analysed HPC changes during the first months after bottling, although few studies have evaluated the HPC after a long period of storage. An increase in the HPC after bottling has been described, reaching a peak after 1-2 weeks. According to different studies,

after reaching this peak, the HPC remains stable at around 4 $\log_{10}$ CFU/ ml after 30 days (Bischofberger and others 1990) or 1 $\log_{10}$ CFU/ ml after 40 days (Urmeneta and others 2000) in glass bottles. Other studies analysing water in plastic bottles have reported a HPC of 7 $\log_{10}$ CFU/ ml at 60 and 180 days after bottling (França and others 2014) and 3 $\log_{10}$ CFU/ ml (Otterholt and Charnock, 2011) or 5 $\log_{10}$ CFU/ ml (Defives and others 1999) at 90 days. In this study, no such large differences were detected. At 365 days, near the expiration date in some brands, the HPC remained between 2 and 3 $\log_{10}$ CFU/ ml. This is in accordance with a study by Bischofberger, in which even after 2 years, the HPC showed values of 3 $\log_{10}$ CFU/ ml (Bischofberger and others 1990) in glass bottles.

Population diversity and similarity indexes

In total, 598 isolates were isolated from all the batches and brand waters during the two year study and were biochemically phenotyped using PhP-48 plates. 97 isolates (16%) showed too little growth and the PhPWin® software removed them before the diversity and similarity analysis to avoid reproducibility problems. Therefore, 501 isolates were finally used for population diversity analyses.

The Simpson diversity indexes (Di) showed a different pattern over time for each batch of water (Table 1). The majority of time points analysed had low diversity values, indicating that there were a few dominant phenotypes among the isolated strains at each point.

From 55 to 125 days, the Di increased in brand batches A1 and B1, whereas it decreased in the second batch of both brands. From 125 days to 365 days, the Di decreased in A2 and B1. For batch B2, the Di stabilised until 365 days, whereas the Di in batch A1 increased. This high diversity index observed in A1 at 365 days could be due to the low number of isolates recovered at that moment. Interestingly, the diversity in brand B was related to HPC fluctuations, since the increases and decreases in the HPC coincided with those of the Simpson diversity indexes. In general, diversity decreased after one year in the bottle since diversities were lower than the initial value in all cases.

Regarding the similarity of subpopulations in brand A, the low pairwise Sp indexes between the subpopulations studied (Sp<0.02) (Figure 1) indicated that the structure and composition of the heterotrophic bacteria varied over time, but also between batches from consecutive years. Notably, subpopulations in the different brand A batches clustered more closely than those in brand B. The bacterial structure was considered similar in samples A1.365 days and A2.125

days, which had a Sp index of 0.25. A Sp analysis of brand B batches showed that the heterotrophic subpopulations from the second batch (B2) were more closely related among them than with subpopulations from the first batch (B1). However, the subpopulations from batch B2 were not considered significantly similar, since the pairwise Sp indexes were below 0.2.

The bottled mineral water studied showed alternating periods of increase and decrease in HPC and diversity. This indicates that each bottle formed a unique micro-ecosystem that evolved differently over time, although all the bottles from the same batch were filled on the same date and were maintained under the same storage conditions. A succession of microbial communities might have occurred and thus, Sp values were lower between subpopulations from the same batch at the different points analysed.

Genotypic characterisation

Seventy eight per cent of the isolates (393 isolates) were grouped in 31 phenotypic groups, each group including the isolates with a similarity equal to or higher than 0.95. The representative isolates of each group formed by more than 2 isolates were selected for further characterisation of the 16S rRNA gene. As a result, nearly 76% (383 isolates) of the isolates were assigned to a taxonomic group (Table 2), providing a representation of the mineral water composition over time. These isolates belonged to 14 different genera. Table A shows the identification of the representative isolates of the phenotypic groups based on the closest relative strain (identity >97%) available on the NCBI 16S ribosomal RNA sequences (Bacteria) database.

There was a clear predominance of *Alphaproteobacteria* (77%) in the isolates from the present study. The remaining isolates were divided into *Gammaproteobacteria* (20%) and *Actinobacteria* (3%). *Alphaproteobacteria* was represented by members of the order *Caulobacterales*, *Rhizobiales* and *Sphingomonadales*. Meanwhile, *Gammaproteobacteria* isolates were identified as *Pseudomonadales* or *Xanthomonadales*. Finally, all *Actinobacteria* isolates belonged to the order *Actinomycetales*.

In the first batch of brand A (A1), the microbial community at 55 days was dominated by isolates belonging to *Sphingomonas* sp., followed by isolates belonging to *Brevundimonas* sp. At 125 days, the dominant genera were *Rhodopseudomonas* and *Sphingopyxis*, while the genera recovered at the first point were not detected. However, at 365 days, the microbial community was similar at genus level to the community at 55 days, but in different proportions. In the second batch (A2), the genus *Blastomonas* was found to be the main group, followed by *Brevundimonas* and *Phenylobacterium* at 55 days. The same genera were recovered at 125 days,

while after one year the genus *Bosea* dominated the community. The genera identified in brand A, *Brevundimonas, Bosea, Rhodopseudomonas* and *Sphingomonas* are common in mineral water since they have already been described in a number of studies (Casanovas-Massana and Blanch 2012; Daood 2008; Falcone-Dias and others 2012; Jayasekara and others 1999; Koskinen and others 2000; Otterholt and Charnock, 2011). To our knowledge, the *Sphingopyxis* genus has not previously been isolated from natural mineral waters, although it has been isolated in fresh water (Baik and others 2013) and sea water (Yoon and Oh 2005; Yoon and others 2005). Similarly, isolates belonging to the *Blastomonas* genus have been identified in water distribution systems (Hwang and others 2012; Revetta and others 2010; Tokajian and others 2005).

In brand B, batch B1 at 55 days, the *Methylobacterium* genus notably dominated the community. This genus contains species considered opportunistic pathogens of immunocompromised patients. These facultative methylotrophs have also previously been recovered from mineral water (Falcone-Dias and others 2012; Jayasekara and others 1999; LeChevallier and others 1980; Otterholt and Charnock, 2011). At 125 days, the predominant isolates belonged to *Mycobacterium* and *Rhodopseudomonas* genera, replacing *Methylobacterium* as the dominant isolates. Many environmental isolates of fast growing *Mycobacterium* genus are considered non-pathogenic and they have been isolated in boreholes and in mineral water a few days after bottling (Casanovas-Massana and Blanch, 2012; França and others 2014). Nevertheless, one year after bottling, the genus *Pseudomonas* was the only genus identified among isolates. In the second batch analysed, B2, the community consisted of isolates belonging to *Afipia, Caulobacter, Phenylobacterium* and *Pseudomonas,* with a preponderance of *Pseudomonas* isolates. *Afipia, Agrobacterium* and *Caulobacter* have been detected in mineral water in the first days after bottling (Falcone-Dias and others 2015; Loy and others 2005; Otterholt and Charnock 2011). *Phenylobacterium* was detected in both brands and it has been detected in boreholes (França and others 2014) and after bottling (Otterholt and Charnock 2011). At the subsequent points analysed, 125 and 365 days, *Phenylobacterium* and *Pseudomonas* isolates were still recovered. Two new genera, *Agrobacterium* and *Pseudoxanthomonas,* which had not been detected at 55 days, were present at 125 and 365 days, and at 365 days, respectively. To our knowledge, *Pseudoxanthomonas* sp. has previously been detected in hot springs (Chen and others 2002) and soil (Li and others 2014; Yoo and others 2007), but not in natural mineral water. *Pseudomonas* spp. are widely distributed in nature due to their ability to grow on a wide range of organic substrates and they are frequently isolated in mineral waters (Casanovas-Massana and Blanch 2013; Falcone-Dias and others 2015; Spiers and others 2000; Urmeneta and others 2000; Vachee and others 1997).

Overall, genotypic characterisation of the isolates confirmed the differences in bacterial populations between different brands and batches of the same brand. The different batches shared only two genera, *Brevundimonas* and *Pseudomonas*, in brands A and B, respectively. However, they were not detected throughout the study period, indicating a fluctuation in populations throughout shelf-life. We anticipated that differences in heterotrophic composition would exist between brands, as this has been extensively reported in previous studies (Hunter 1993; Loy and others 2005; Mavridou 1992; Venieri and others 2006). Differences between batches collected in consecutive years have been also observed, which may be attributed to various reasons. For example, microbial communities may not be evenly distributed in the underground water table, and variations in the catchment point may be responsible for such differences. Alternatively, differences in bottling procedures may introduce changes in environmental conditions, leading to the development of a different heterotrophic community. However, we cannot exclude the possibility that the communities were similar after bottling, since this study did not focus on the early stages. After bottling, the succession of microbial communities in the bottles may be driven by their different ability to survive in an oligotrophic and closed environment. Some species may grow at the expense of organic matter from lysed species or as a result of the reactivation of starving cells initially present (Leclerc and Moreau 2002).

Conclusions

To sum up, we found that bottled mineral water contains complex and diverse microbial communities. These communities are different between brands and they change during the shelf-life of the bottles, which leads to different dominant bacterial species after the mineral water has been inside the bottle for a long time. Moreover, we did not observe a generalisable trend that explained the behaviour of the bacterial heterotrophic community in mineral water inside a bottle using culture-dependent methods. In addition, despite fluctuations in diversity, the overall trend was a decrease in diversity after one year in the bottle. Further studies are needed not only to determine the factors responsible for fluctuations in heterotrophic populations during shelf-life, but also to assess whether the changes in composition may pose a risk for human consumption.

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Author contributions

Laura Sala-Comorera performed the experiments, analyzed the data and prepared the manuscript; Arnau Casanovas-Massana and Cristina García-Aljaro analyzed and interpreted the data; Anicet R. Blanch designed the experiments and supervised the project. All authors read through, made contributions to improve the manuscript, and approved the final version.

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Tables and figures

Table 1. Concentration of aerobic heterotrophic bacteria (HPC) (expressed as average $\log_{10}$ CFU/ml ± standard deviation) and diversity indexes (Di) for each subpopulation. The number of isolates analysed in each batch is indicated in brackets. A1: mineral water A first batch, A2: mineral water A second batch, B1: mineral water B first batch, B2: mineral water B second batch.

	Bottle A1		**Bottle A2**		**Bottle B1**		**Bottle B2**	
	HPC	*Di*	HPC	*Di*	HPC	*Di*	HPC	*Di*
55 days	3.62 ± 0.03	0.59 (43)	3.60 ± 0.03	0.87 (26)	2.76 ± 0.05	0.44 (49)	3.74 ± 0.10	0.81 (50)
125 days	3.76 ± 0.04	0.57 (49)	2.88 ± 0.09	0.80 (47)	3.25 ± 0.09	0.97 (50)	3.26 ± 0.04	0.67 (48)
365 days	1.99 ± 0.17	0.75 (22)	2.68 ± 0.05	0.58 (47)	2.54 ± 0.10	0.35 (35)	3.00 ± 0.08	0.67 (35)

Table 2. Heatmap representing the genera and species identified in each brand over time.

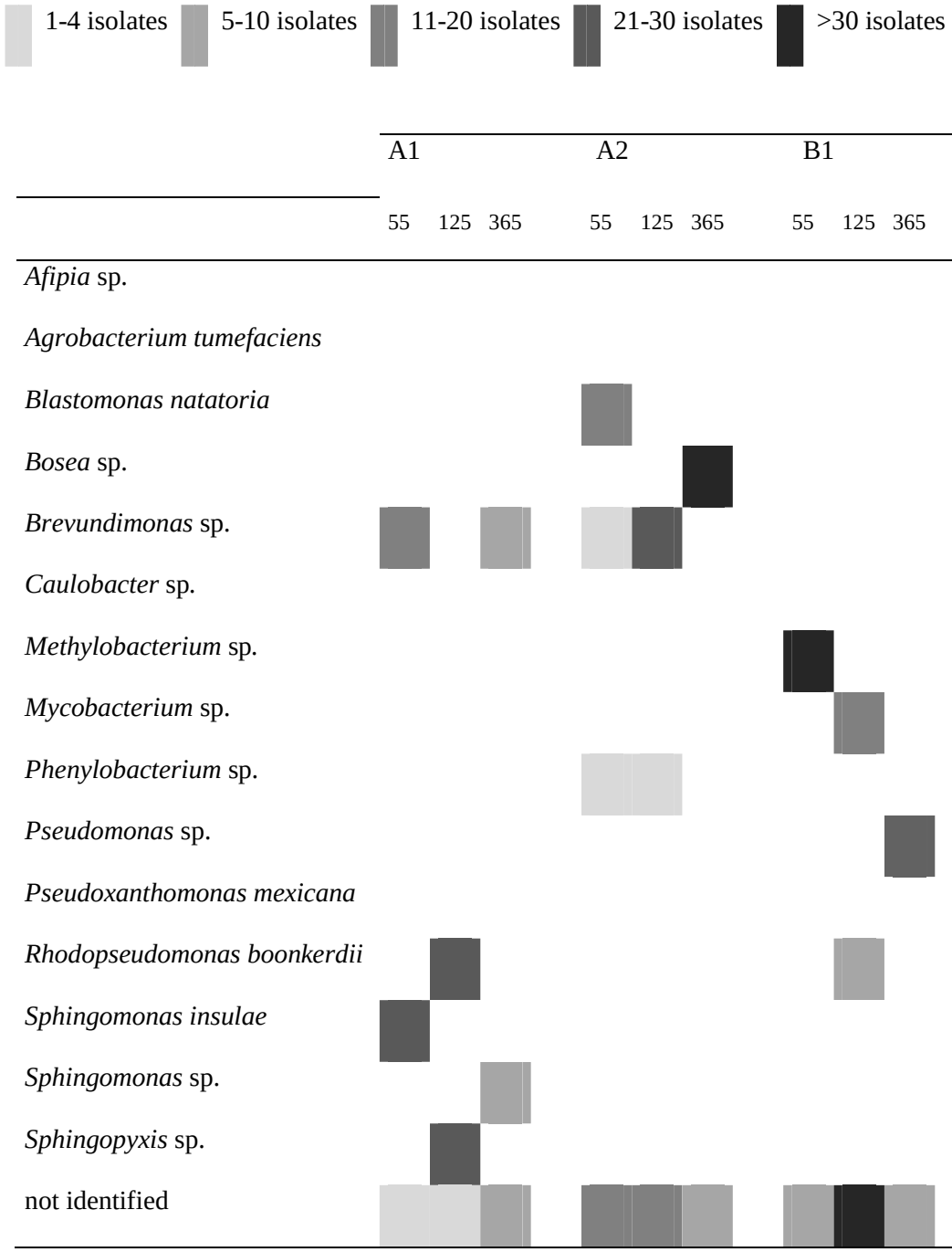
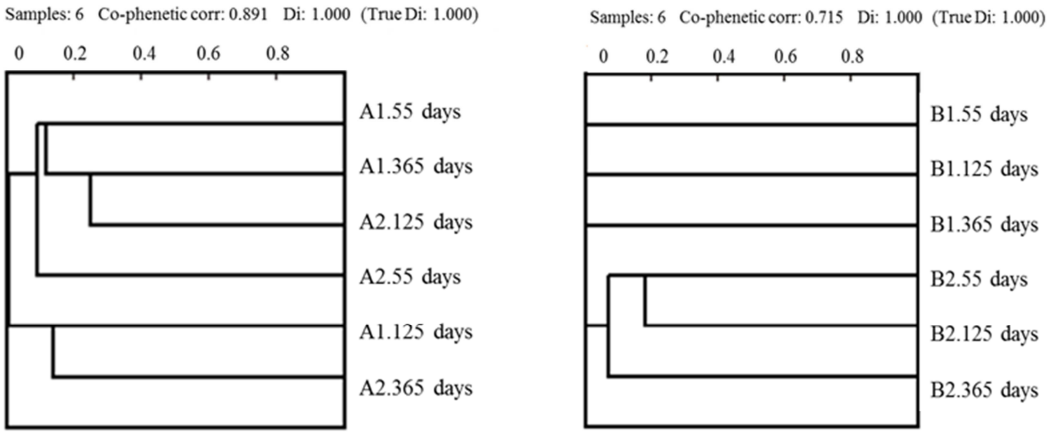


Figure legend

Figure 1. Clustering of the population similarity indexes (Sp) for the two mineral waters at 55, 125 and 365 days. A1: mineral water A first batch, A2: mineral water A second batch, B1: mineral water B first batch, B2: mineral water B second batch.



Supplemental material

Table A. Identification of most representative strains and percentage of similarity to the closest relative strain at the NCBI database.

Isolate	Accession no.	Closest relative and its accession no.	Number of strains in the cluster
A1.21.1	KX442615	*Brevundimonas* sp. NR_113586.1 NR_028633.1 NR_037104.1	17
A1.19.1	KX442616	*Sphingomonas insulae* NR_044187.1	4
A1.22.1	KX442617	*Sphingomonas insulae* NR_044187.1	25
A1.48.2	KX442618	*Rhodopseudomonas boonkerdii* NR_114302.1	21
A1.17.2	KX442619	*Sphingopyxis* sp. NR_112561.1	24
A1.39.3	KX442620	*Brevundimonas* sp. NR_037108.1	10
A1.16.3	KX442621	*Sphingomonas* sp. NR_113867.1	5
B1.49.1	KX442622	*Methylobacterium* sp. NR_041025.1	3
B1.15.1	KX442623	*Methylobacterium* sp. NR_112233.1	39
B1.27.2	KX442624	*Rhodopseudomonas boonkerdii* NR_114302.1	7
B1.39.2	KX442626	*Mycobacterium* sp. NR_025393.1	7
B1.15.2	KX442627	*Mycobacterium* sp. NR_025393.1	4
B1.29.3	KX442628	*Pseudomonas* sp. NR_117821.1	30
A2.28.1	KX442629	*Afipia sp.* NR_029200.1	5
A2.41.1	KX442630	*Blastomonas natatoria* NR_113794.1	10
B2.34.2	KX442631	*Phenylobacterium* sp. NR_041991.1	33
A2.44.1	KX442632	*Brevundimonas* sp. NR_037108.1	25
A2.1.3	KX442633	*Bosea* sp. NR_028799.1	32
A1.19.2	KX442634	*Brevundimonas* sp. NR_037108.1	3
A2.8.3	KX442642	*Bosea sp.* NR_028799.1	5
B2.6.1	KX442635	*Pseudomonas* sp. NR_042541.1	18
B2.38.1	KX442636	*Pseudomonas* sp. NR_113600.1	20

B2.9.1	KX442637	*Pseudomonas* sp. NR_025103.1	3
B2.29.1	KX442638	*Caulobacter* sp. NR_074208.1	4
B2.32.3	KX442639	*Agrobacterium tumefaciens* NR_041396.1	23
B2.18.3	KX442641	*Pseudoxanthomonas mexicana* NR_113973.1	6

4.2. Article 2

Evaluation of the bacterial community to assess the traceability of bottled water of different mineral water brands during the shelf life

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Les aigües minerals naturals són ecosistemes complexos des d'un punt de vista fenotípic i genotípic i contenen una comunitat microbiana pròpia. La comunitat bacteriana dins l'ampolla evoluciona durant el temps i es produeixen increments en el nombre de microorganismes heteròtrofs. Els motius que expliquen la multiplicació dels microorganismes després de l'envasat i les successions poblacions que tenen lloc, encara estan subjectes a debat. La reactivació de cèl·lules en estat de latència, els components orgànics que es desprenen de l'envàs o l'efecte de l'ampolla són factors que poden contribuir a l'increment del nombre d'heteròtrofs. A més a més, el material de l'ampolla es creu que pot tenir un efecte sobre el desenvolupament de la comunitat bacteriana, així i tot, pocs estudis han valorat l'efecte del material.

L'objectiu d'aquest estudi va ser valorar si la comunitat bacteriana present a l'aigua mineral natural envasada era constant durant el temps de vida comercial per a utilitzar-la com a traçador de l'aigua mineral. Amb aquesta finalitat, s'utilitzaren mètodes dependents de cultiu (recompte d'heteròtrofs en placa) i no dependents de cultiu (recomptes de cèl·lules amb colorants vitals i l'electroforesi en gel amb gradient desnaturalitzant (DGGE)).

Per això, es van seleccionar 3 aigües minerals naturals, cada una d'elles envasada en el mateix dia amb ampolles de vidre i polietilè tereftalat (PET). Les ampolles van ser emmagatzemades a la foscor en una cambra a 22 ˚C i van ser analitzades a 1, 7, 15, 21, 30, 45, 60 i 90 dies després de l'envasat. Els recomptes d'heteròtrofs (cèl·lules cultivables) en placa es van realitzar mitjançant filtració a través de membranes d'un porus de 0,22 µm de diàmetre i incubades en medi R2A. El test de viabilitat Live/dead® BacLight™ (Invitrogen) es va utilitzar per a l'enumeració de bacteris totals, després de filtrar diferents volums d'aigua per un filtre de 0,22 µm de diàmetre, es van observar amb un microscopi d'epifluorescència. Per a l'anàlisi mitjançant DGGE, es van filtrar 3 litres d'aigua a través de

membranes de policarbonat de 0,22 μm de diàmetre de porus. L'extracció d'ADN de les membranes es va realitzar mitjançant l'acció mecànica de partícules de vidre i la purificació amb fenol i cloroform. La zona variable V3 del gen ARNr 16S es va amplificar amb una PCR imbricada i la DGGE es va realitzar a 200 V durant 5 h amb un gel de poliacrilamida amb un gradient desnaturalitzant del 35-70% amb un contingut del 8% d'acrilamida. Finalment, les bandes del gel rellevants van ser extirpades i clonades en un plasmidi p-GEM®-T Easy Vector Systems (Pronadisa) i seqüenciades. Es va realitzar el test estadístic Jackknife i l'anàlisi de components principals (PCA) i es va calcular la diversitat de Shannon. També es va adquirir una ampolla de cada marca i material d'un lot diferent a l'estudiat en establiments comercials i es van comparar els seus perfils moleculars amb els perfils del lot analitzat durant 3 mesos.

Els resultats obtinguts van revelar que els recomptes totals de cèl·lules van ser al voltant de 10^3 cèl·lules/ml en totes les ampolles i marques després de l'envasat. Un 10% del total de cèl·lules eren viables mentre que els recomptes d'heteròtrofs van representar menys del 0,1% de la comunitat. Entre la primera i la segona setmana després de l'envasat, el recompte de cèl·lules totals i viables va augmentar 1-2 unitats $\log_{10}$. Les aigües envasades en ampolles de vidre van assolir concentracions majors de viables respecte les ampolles de PET, encara que l'increment es va produir més lentament. Els recomptes totals de cèl·lules van incrementar fins a 10^4-10^5 cèl·lules/ml i van restar constants durant els 3 mesos, com també les cèl·lules viables que van assolir concentracions de 10^3-10^4 cèl·lules/ml. Un dia després de l'envasat, els recomptes de cèl·lules cultivables van ser molt baixos < 50 UFC/ml i en l'aigua mineral B no es van detectar. Després de l'envasat, van experimentar augments de 3-4 unitats $\log_{10}$ durant els 15-21 dies. Les cèl·lules cultivables després d'augmentar i assolir una concentració màxima durant les primeres setmanes van oscil·lar fins als 3 mesos. Les ampolles de vidre van reportar concentracions més altes de cèl·lules cultivables que les respectives en PET. Tot i així, a partir del dia 21 i fins el dia 90, no es van observar diferències significatives entre els recomptes de cèl·lules totals, viables i cultivables observats entre les ampolles de PET i vidre. Les empremtes moleculars obtingudes van ser semblants per a la mateixa marca d'aigua mineral 24 hores després de l'envasat, a partir d'una setmana les empremtes moleculars es van diferenciar respecte la mostra del dia 1 però també entre la mateixa marca d'aigua envasada en PET i vidre. Aquelles marques que es van diferenciar més ràpidament respecte de l'empremta molecular inicial, van mantenir una empremta molecular constant durant els 3 mesos, en comparació amb les respectives aigües envasades en l'altre material. Les empremtes moleculars es van unir en el dendrograma en funció de la marca i material de l'envàs,

formant grups separats. De fet, van preservar la seva pròpia identitat fins als 3 mesos després de l'envasat. El material de l'envàs podria afectar el desenvolupament de la comunitat, ja que les empremtes moleculars obtingudes van ser diferents en les ampolles de PET i vidre de la mateixa aigua mineral natural envasades el mateix dia. L'anàlisi PCA va permetre separar certes aigües, a més, les ampolles seleccionades a l'atzar presentaven algunes bandes comunes a les ampolles analitzades durant els 3 mesos. En conseqüència i atès que les aigües van tenir una empremta molecular característica, es suggereix la possibilitat de definir marcadors moleculars per a la seva traçabilitat.

Evaluation of the bacterial community to assess the traceability of bottled water of different mineral water brands during the shelf life

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Abstract

Natural mineral waters typically contain low concentrations of indigenous bacteria that are usually characteristic of each spring. Once bottled, these communities may change over time until the water is consumed. The bottling material is believed to play a major role in the succession of these populations, but until date, very few studies have evaluated the bottle effect in the bacterial communities. The microbial community structure of three natural mineral water brands were investigated over 3 months after bottling in glass and PET bottles for their traceability. To this end, culture-dependent (heterotrophic plate count) and culture-independent methods (PCR-DGGE (polymerase chain reaction -denaturing gradient gel electrophoresis) and fluorescent microscopy with vital dyes) were used. The total and viable cell counts increased around 1-2 $\log_{10}$ units between one and two weeks after bottling and remained constant during the 3 months analyzed regardless of the bottling material. The highest increase in viable cells occurred in glass-bottled waters, however the increase was one week later than in the PET-bottled waters. The culturable counts fluctuated during the study, in average they accounted for less than 10% of the total cell counts. The DGGE fingerprints obtained indicated that a different community had been established two weeks after bottling in the different bottling material and brands. Shifts in bacterial composition at different times during the 3 months were observed, however the cluster analysis of the DGGE fingerprints revealed that the samples clustered mainly according to the brand and the bottle material. In conclusion, no difference in the number of total, viable and culturable bacteria counts were observed among mineral water bottled with PET and glass during long term storage. Some of the water brands and/or material had a distinct microbial community structure clearly distinguishable from the others, which suggests the possibility of defining a molecular marker for traceability.

Introduction

Natural mineral waters are not sterile environments, but complex ecosystems with a high phenotypic and genetic diversity that have been reported in different studies (Casanovas-Massana and Blanch, 2012; Loy et al., 2005; Rosenberg, 2003). Mineral water according to the Directive 2009/54/EC cannot be subjected to any disinfection or bacteriostatic treatment so as to modify the microbiota. Therefore, natural mineral waters contain their autochthonous microbiota, which is clearly distinguishable from other mineral water coming from different origin (Casanovas-Massana and Blanch, 2012; Hunter, 1993; Loy et al., 2005; Mavridou, 1992; Venieri et al., 2006). Natural mineral water means microbiologically wholesome water, the Directive states the absence of microbial parameters such as faecal indicators: *Escherichia coli* and other coliforms, faecal streptococci and *Pseudomonas aeruginosa* in any 250 ml of sample examined, and sporulated sulphite-reducing anaerobes in 50 ml, and free from parasites or pathogenic microorganism at source and after bottling. As a result, both at the source and the water bottling plant have to be strictly controlled and monitored to prevent any contamination that may pose a risk to consumer health (Hrudey et al., 2006). The Directive also states that in the finished product, the total aerobic heterotrophic colony count (HPC) 12 h after bottling cannot exceed the number of 100 CFU mL^{-1} after an incubation for 72 h at 20-22 ºC and 20 CFU mL^{-1} after an incubation for 24 h at 37 ºC.

The number of heterotrophic bacteria colonies is the most commonly used criteria for microbiological quality assessment. However, there is no evidence in clinical and epidemiological research to link the HPC with the influence on the human health. Many literature sources describe a rapid growth of the microorganism in bottled water samples after the bottling process (Diduch et al., 2016; Falcone-Dias and Farache Filho, 2013; Urmeneta et al., 2000). Some authors have reported an exponential increase of HPC within several days after bottling, which reaches a peak after 1-3 weeks after bottling (Bischofberger et al., 1990; Hunter, 1993; Urmeneta et al., 2000). Afterwards, the HPC maintains constant or decreases slightly until one year after bottling (Bischofberger et al., 1990). The HPC during storage fluctuates, in fact, there is not a general trend to explain the behaviour of the HPC inside the bottle during their shelf-life (Falcone-Dias et al., 2012).

The reasons for the bacterial multiplication after bottling and the succession of the community are still not fully understood. There is some controversy concerning the factors, which influence the increase of the heterotrophic populations. The concept of “bottle effect” in which the ratio of the surface area to volume promote the bacterial growth originally proposed by Zobell and Anderson (Zobell and Anderson, 1936) was refused by Hammes (Hammes et al., 2010), thus contradicting numerous previous reports. It has been suggested that the concentration of organic

matter available, the large amounts of bicarbonate and the total dissolved solids serve as material for the multiplication of the microorganisms (Falcone-Dias and Farache Filho, 2013) until the organic material in the water has been depleted (Rosenberg, 2003). In fact, there is a clear correlation between HPC number and the concentration of assimilable organic carbon in bottled mineral water (Diduch et al., 2016). The bacterial regrowth could be explained as a result of reactivation of starving cells initially present (Leclerc and Moreau, 2002). Moreover, during the shelf-life a succession of the microbial communities has been reported, which could grow at the expense of organic matter from dead cells of the former population (Falcone-Dias and Farache Filho, 2013).

The bottling material is believed to play a major role in the succession of these populations, but until date, few studies have evaluated the effect of the material in the bacterial communities. Some studies revealed that the low molecular substances migrating from PET and PVC plastic could promote the growth of bacterial populations. In contrary residual cleaning agents, whether bottles are reused, could interfere in the bacterial populations, yielding a bacteriostatic effect (Bischofberger et al., 1990). Moreover, the colour of the packing material affect the microorganism content, slower colony count were found in transparent PVC bottle in comparison to dark glass bottle, in which the colour of the bottle protect bacteria from the daylight (Mavridou, 1992). Currently, polyethylene terephthalate (PET) bottles are the most widely used material bottling water due to its properties: low weight, resistant, colourlessness and transparency, resistance to chemicals, strength, flexibility, impact-resistance and easy recycling procedures (Spangenberg and Vennemann, 2008; Welle, 2011). PET bottles have replaced glass bottles, which have been mainly displaced to hotel and restoration sector (personal communication from water companies).

Although the regulations focus only in the cultivable bacteria fraction in mineral water (Anonymous, 2009), mineral water contains an important fraction which is not cultivable under the established cultivation parameters and it is metabolically active (França et al., 2014; Loy et al., 2005). Thus, HPC, which are routinely determined, underestimates the total number of microorganisms present in the mineral water. For this reason, culture independent methods offer a more accurate description of the microbial community dynamics overtime inside the bottles (França et al., 2014; Vaz-Moreira et al., 2011). Among culture independent methods, DGGE has been successfully used to generate a community fingerprint in a particular moment and to study the dynamics of the community overtime (Burtscher et al., 2009; Dewettinck et al., 2001).

The aim of this work was to assess if the microbial communities present in a given mineral water brand remain constant over time during their shelf life for their traceability. To this end, the communities present in three different brands of mineral water bottled in two different material (glass and PET) were analysed by culture-dependent (heterotrophic plate count) and culture-independent methods (PCR-DGGE and fluorescent microscopy with vital dyes).

Materials and methods

Water samples

Three bottled mineral water brands from independent Spanish springs (A, B and C) were assessed. Each mineral water was bottled on the same day from the same spring in glass and PET bottles (1.0-1.5 litre bottles). From each brand and material, 35 bottles from the same batch were taken directly from the end of the filling line. Bottles were stored under dark conditions at 22 °C and samples were processed on days 1, 7, 15, 21, 30, 45, 60 and 90 after bottling.

A bottle for each brand and material from a different batch were purchased randomly in a retailer to compare their molecular profiles with the batch analysed for 3 months.

Enumeration of total bacteria

The bacterial viability was assessed with LIVE/DEAD® (L/D) BacLight™ (Invitrogen, USA). Briefly, volumes ranging from 10 ml to 50 ml were filtered through 0.22 µm pore size black polycarbonate membrane filters (Millipore, German). Filters were covered with 1 ml deionized sterile water containing 3 µl L/D stain mixture and incubated at 37 ℃ in the dark for 15 min. Afterwards filters were dried in the dark at room temperature and observed under an epifluorescence microscope (Leica Microsystem, Germany) equipped with a mercury lamp. A minimum of 20 fields selected at random were counted at 1008x. Only green fluorescent cells were considered to be alive, other cells were considered to be dead (Haugland, 1996).

Enumeration of cultivable heterotrophic bacteria

For aerobic and facultative anaerobic heterotrophic enumeration, samples ranging from 10 µl to 500 ml were filtered through 0.22 µm pore size polycarbonate membrane filters (Millipore, Germany), in triplicate. Filters were incubated on R2A plates (Pronadisa, Spain) for 7 days at 20 ± 2 ℃ and viable colonies were enumerated.

DGGE analysis

DNA extraction and PCR amplification

Three litres were filtered from each sample through 0.22 µm pore size polycarbonate membrane filters (Millipore, German). DNA was extracted from the filters applying mechanical disruption through bead beating and phenol/chloroform purification using a modification of a previous protocol (Griffiths et al., 2000). Extraction was performed by the addition 0.37 g (≤106 µm) glass beads acid washed, 0.25 g (1 mm) glass beads acid washed, 1 piece (3 mm) glass beads acid washed, 400 µl chloroform/isoamyl alcohol (24:1 v/v), 400 µl phenol and 400 µl CTAB-buffer (100mM TRIS HCl pH 8, 1.4M NaCl, 20mM EDTA, 2% (w/v) CTAB, incubated for 30 min at 75℃ and then adding 0.2% (v/v) mercaptoethanol). Samples were lysed for 15 min, speed 10 with Vortex Genie® 2 (Scientific Industries Inc, USA), and immediately were chilled on ice (1 min). The aqueous phase was separated by centrifugation (16,000 × g) for 5 min and mixed with 500 µl chloroform/isoamyl alcohol l (24:1 v/v) followed by a centrifugation (16,000 × g) for 5 min. Supernatant were transferred into a vial with 0.6 volumes of isopropanol and incubated at room temperature overnight and followed by a centrifugation (16,000 × g) for 10 min at 4^{o}C. Pelleted nucleic acids were then washed twice in ice cold 70% ethanol and air dried prior to being resuspended in 20 µl in TRIS pH 8 10mM.

The V3 hypervariable region from 16S rRNA gene was amplified by nested PCR. In the first step, primers 27f (5' -AGA GTT TGA TCM TGG CTC AG- 3') and 1492r (5' -TAC GGY TAC CTT GTT ACG ACT T- 3') (Weisburg et al., 1991) were used. The PCR was performed in a total volume of 50 µl including 25 µl of DreamTaq Green PCR Master Mix (2x) (Thermo Scientific, USA), 0.5 µM of each universal bacterial primers and 2 µl of DNA. PCR conditions were: 94 ℃ for 5 min, 35 cycles of 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1.5 min. A final extension phase of 72℃ for 7 min was used. In a second step, the samples were reamplified using the pair of primers PRBA338f (Lane, 1991; Ovreas et al., 1997) (5' -ACT CCT ACG GGA GGC AGC AG- 3') with a GC clamp to the 5' end (5' -CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G- 3') and PRUN518r (Muyzer et al., 1993) (5'-ATT ACC GCG GCT GCT GG- 3'). Each 100 µl of PCR mixture contained 50 µl of DreamTaq Green PCR Master Mix (2x) (Thermo Scientific, USA), 0.1 µM of each universal bacterial primers and 5 µl of DNA of PCR product. The nested-PCR was performed under the following conditions: 94℃ for 4 min; 30 cycles of 94℃ for 1 min, 59℃ for 1 min, and 74 ℃ for 1 min; and 74℃ for 3 min.

DGGE analysis of PCR products

DGGE was performed with a DCode system (Bio-Rad, USA) as previously described (Ballesté and Blanch, 2011). Electrophoresis was performed with 1-mm thick 8% (w/v) polyacrylamide gels (30% acrylamide/bis-acrylamide [37.5:1]. About 800 ng of nested-PCR product were loaded into lanes of a gel, containing a linear 35-70% denaturing gradient (100% denaturant agent was defined as 7 mol/L urea and 40% [v/v] formamide). Nested-PCR product were quantified by electrophoresis with a 2% agarose gel using 4 µl of low DNA Mass Ladder (Invitrogen, USA) and stained with ethidium bromide and visualized by fluorescing when exposed to UV light. For comparison among different DGGE gels, a DGGE-marker was used, consisting in four strains isolated by our research group from mineral water or drinking water (T32.2- *Micrococcus* sp., V44.2-*Sphingopyxis* sp., 3B.B24-*Pseudomonas* sp. and TR11.2-*Bacillus* sp.). They were cultured on Tryptic Soy Broth (Pronadisa, Spain) at 20 ± 2 ºC for 24 ± 2 h. DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Spain) following manufacturer's instructions. A total of 10 µl of a mixed of nested-PCR product from the reference strains were loaded into 3 lanes in each gel.

The gel was run for 15 min at 20 V followed by 5 h at 200 V in 1x Tris-acetate acid-EDTA (TAE) (40 mmol/L Tris, 20 mmol/L sodium acetate, 1 mmol/L EDTA; pH = 7.4) at 60 ^{o}C. Gels were visualized by 45 min staining in 1x sodium chloride-Tris-EDTA buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA; pH 7.4) with SYBRGold nucleic acid stain (Molecular Probes Inc.,USA), followed by a subsequent analysis under UV radiation with ChemiDoc^{TM} MP Imaging System (BioRad, USA). Gels were scanned using the Quantity One 4.6.7 program (Bio-Rad, USA).

The positions and relative signal intensities of detected bands in each gel track were determined with FPQuest Software v.5.10 (Bio-rad, USA). Cluster analysis of DGGE patterns was performed using FPQuest Software v.5.10. Normalisation was achieved by a reference pattern of an inter lane standard applied to every 1st, 6th and 11th lane on the DGGE gels. For cluster analysis, unweighted-pair group method analysis (UPGMA) and Dice distance were used and 1% of position tolerance of the bands and 1% optimization was accepted. Following DGGE analysis, Jackknife analysis was used to determine how accurately DGGE fingerprints of bottled water communities could be assigned to each water brand. To perform Jackknife analysis, bottle water community fingerprints were manually assigned to their respective brand and material group. The software then removed each fingerprint from the data set individually and queried the data set to determine from which brand and material group the fingerprint was most similar. User-set parameters included maximum-similarity coefficients and ties spread equally among

groups. The internal accuracy of classification was calculated as the percentage of community fingerprints assigned to the group to which the sample was known to belong.

Extraction of DNA from DGGE bands and sequencing

Common bands were excised and were transferred into a vial containing 30 µl of ultra-pure water and stored at 4^{o}C overnight. A total of 5 µl was used for reamplified with the primers PRBA338f and PRUN518r with the same conditions mentioned above. PCR products were cloned into p-GEM®-T Easy Vector Systems (Pronadisa, Spain) according to manufacturer's instructions and Sanger sequenced (ABI Prism 3700; PerkinElmer, Thermo Fischer Scientific, USA). The 16S rRNA gene sequences were submitted for similarity searches to the National Center for Biotechnology Information using Blast search tool (http://www.ncbi.nlm.nih.gov/Blast/).

Statistical analyses

Principal component analysis (PCA) of the DGGE fingerprints was performed to analyse temporal variations of the bacterial community structure based on the relative band intensity and positions using GelCompar II Software (Applied Maths, Belgium). Differences in the cell counts between PET and glass bottles were performed using Mann-Whitney test with the median concentrations.

Diversity analyses

The Shannon-Weaver index (H') was used as an estimate of microbial diversity (Tiodjio et al., 2014).

Results

Total and cultivable heterotrophic bacteria counts

Changes in the number of total, viable and culturable cells of the 3 mineral water brands examined are shown in Fig. 1. In brand A, total cell counts in PET- and glass-bottled water were similar, from this point, the total cell counts increased, reaching a peak two weeks after bottling in both materials. The initials viable bacterial counts in both materials were nearly $1x10^3$ cell/ml and they increased around 2 $\log_{10}$ units between days 1 and 15. Afterwards, the viable bacteria remained in the range of $8.9x10^3$ to $4.7x10^4$ cell/ml until day 90 in glass-bottled water. While the viable bacteria in PET-bottled water remained in the range of $2.0x10^4$ to $2.9x10^4$ cell/ml until day 60. Between days 60 and 90, the total cell counts and the viable bacteria increased. However, no differences were reported in viable counts from day 21 to day 90 between PET- and glass-bottled water ($p=0.06$). One day after bottling, the culturable bacteria represented a minor part of the total number of cells in both materials, less than 1% of the population, and between day 7 and 15 reached a maximum in both materials. Afterwards, the culturable counts decreased until one month after bottling, from this point, the culturable counts showed an upward trend until the end of the experiment in glass-bottled water, whereas in PET-bottled water, the culturable counts remained at the same level until day 60. Anyway, the culturable counts in A PET-bottled water were lower than A glass-bottled water in all the points tested from day 15, however, no significant differences were reported ($p=0.38$).

The initial total cell values and viable cells in brand B were similar in both materials and both counts increased 1 $\log_{10}$ unit within 7 days after bottling. The total and viable counts in B PET-bottled water decreased until day 21 after the sharp increase in a week. Afterwards, they showed an upward trend until the end of the period analysed in PET-bottled water. The viable counts in PET-bottled water ranged from 15 to 32% of the total cell counts from day 15 to day 90. On the other hand, the total counts and the viable counts remained stable for 3 months in B glass-bottled water; viable counts represented 34-82% of the total cell counts. Culturable heterotrophic counts were lower than 1 CFU/500ml 1 day after bottling in both materials. However, the values of culturable counts in glass-bottled water increased sharply to $6.5x10^3$ cell/ml within 15 days after bottling. The culturable fraction represented 23-35% of the total cell counts from day 15 onward. In B PET-bottled water, the culturable counts reached a maximum of $1.3x10^3$ cell/ml one month after bottling, from this point, the value of culturable cells decreased. Below 1% of the total cell counts were culturable cells, only at day 21 and 30, the culturable fraction accounted for 9% of the total cell counts in PET-bottled water. It should be noted that the percentage of viable cells and cultivable counts in B PET-bottled water were

lower than B glass-bottled water, a large number of damaged cells were present in PET-bottled water. Nevertheless, from day 21, no differences in the total and viable counts among PET and glass were reported ($p>0.05$), whereas significant differences in the culturable counts ($p=0.12$).

The total cell and viable counts in C PET-bottled water increased around 2 $\log_{10}$ units between day 1 and 15. After reaching a peak at 15 days, the total cell counts remained in the range of $3.0x10^4$ to $8.8x10^4$ cell/ml until day 90. In contrast, viable counts reached a peak of $1.4x10^4$ cell/ml at 15 days, and remained in the range of $5.6x10^3$ to $2.0x10^4$ cell/ml until day 90 (25% of the total cell counts). Nevertheless, the viable counts in PET-bottled water accounted for less than 32% of the total cell counts in all the period analysed. Viable cell counts from C glass-bottled water increased to $2.9x10^4$ cell/ml at day 21. From this point, viable cell counts represented 33-49% of the total cells and remained in the range of $1.1x10^4$ to $3.2x10^4$ cell/ml during the analysed period. The initial values of culturable counts in brand C were $6.8x10^1$ CFU/100ml in PET-bottled water and 8 CFU/100ml in glass-bottled water 1 day after bottling. Within 15 days after bottling, the culturable fraction of PET-bottled water increased to $1.3x10^4$ CFU/ml, corresponding to around 10% of the total cell counts. In glass samples, the increase was slower, reaching a maximum culturable counts of $1.7x10^3$ CFU/ml 1 month after bottling. After the peak, a period of fluctuation (decreases and increases) followed onward in both materials. When the culturable fraction reached the maximum values, this represented 22 and 46% at 21 and 60 days respectively in PET-bottled samples and below 5% in glass-bottled sample. From day 21 until the end of the experiment, there was no significant differences in the total, viable and culturable counts among C PET- and C glass-bottled water tested ($p>0.05$).

Analysis of DGGE fingerprints

The molecular fingerprints of the three mineral water brands bottled in PET and glass were compared by DGGE at different days after bottling. A cluster analysis of the matrix of similarity values was then performed and visualised in a dendrogram for the different brands. Each water brand had a distinct DGGE fingerprint which suffered variations at the different days after bottling (Fig.2).

Dendrogram analysis for mineral water brand A showed that the water samples grouped into two main clusters according to the bottle material (Fig. 3A). One day after bottling the

differences in the communities were already evident showing a Dice similarity value of 54% (6 bands out of 18 bands in common between both materials). All the DGGE fingerprints from day 7 onward were very similar (76-96%) in A PET-bottled water (Fig. 2A), each sample had roughly 3 prominent bands and about 10 minor bands, the intensity of which changed. Furthermore, 5 bands were shared by all the A PET-bottled water samples (202, 219, 241, 292, 480). Band 219 was the most prominent of the DGGE profiles from all the PET-bottled water, this band showed similarity to a *Hyphomicrobium* (Table 1). Band 292 belonged to *Rhodococcus* genus and band 480 to *Hydrogenophaga palleronii*, whereas band 241 was associated to an uncultured bacterium. Band 202 could not be extracted due to its low concentration.

Major differences between DGGE fingerprints were observed in A glass-bottled water (Fig. 2B). The fingerprints changed throughout the experiment with similarities of the Dice band-based coefficient values ranging from 65 to 87%. Certain bands were detected during all the experiments (271, 281, 292), while other bands could not be observed in all the samples. These common bands were associated to a different class (*Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* respectively*)* as shown in table 1.

The number of bands visualized in each DGGE fingerprint from A PET- and A glass-bottled water was similar, between 13 and 19 bands were detected in each DGGE fingerprints. Moreover, it was not detected a decrease in the number of bands over time. Band 292 appeared to be common in all the samples from both materials, whereas 5 bands more were also present in 75% of the A brand samples. Band 292 was associated to *Rhodococcus* sp., this *Actinobacteria* was detected only in water A (glass and PET). While this band was prominent at day 1 and 7 in PET-bottled water, the intensity of the band decreased during storage. In contrast, in glass-bottled water, the intensity of this band remained constant.

In brand B, based on the number of DGGE bands, a more diverse community was present at one day after bottling in both materials (21 bands) than the other samples tested (Fig. 2C and 2D). These two samples (B PET 1 and B glass 1) had fingerprints quite similar with a Dice similarity coefficient of 73%. Cluster analysis (Fig. 3B) indicated that samples B PET 1 and B glass 1 formed one separated cluster along with sample B PET 7. Band fingerprints exhibited slight variations between one and seven days of PET-bottled water (B PET 1 and B PET 7) (62% Dice similarity coefficient). Nevertheless, PET-bottled water displayed greater temporal variations during the study from 15 days after bottling, there were not an established community. Cluster

analysis showed low Dice similarity coefficient values ranging from 50 to 70% among B PET-bottled water. The highest similarity in the DGGE patterns were observed between B PET 45 and B PET 90 samples (Dice similarity coefficient = 92%). There were no dominant bands in the DGGE fingerprints, although 5 bands (155, 241, 253, 254, 255) were detected in 6 out of 8 samples. Bands 241 and 255 showed similarity to an uncultured bacterium, whereas band 155 was related to *Porphyrobacter sanguineus.* Band 253 and 254 were associated to genus *Leptospira.*

DGGE fingerprints in brand B from glass-bottled water were more stable than those from PET-bottled water during the three months analysed. Although a significant fingerprint change was observed one week after bottling, (55% Dice similarity coefficient between the sample processed one day after bottling and the sample processed 7 days after bottling), afterwards the DGGE fingerprints remained similar during storage (Fig. 2D). In fact, in brand B glass-bottled water a one well-defined cluster for the whole set of samples analysed was observed with two subclusters (Fig. 3B). The first one included the DGGE B glass-bottled fingerprints at 7, 15, 21 and 30 days which had 4 bands in common, whereas the second group contained the fingerprints of the samples after long-term storage with 5 bands in common. The presence of band 187, 190 and 191 in DGGE fingerprints from samples after long-term storage contributed to the clustering of these samples with the consequence separation from the first samples. Band 476 and 255 were detected in all the 8 samples analysed, and were associated to *Acidovorax radicis* and to an uncultured bacterium associated to the microbiota of the water in a drinking water treatment plant, respectively. Notably, there was a dominant band (170) in all B glass-bottled water samples except in the sample at 1 day after bottling. This band showed similarity to a *Ramlibacter henchirensis* (Table 1).

The number of bands obtained in B PET- bottled water was higher than in B glass-bottled water (9-14 as opposed to 5-12 bands). Two bands were detected in common in both materials (255 and 243), though the intensity of the bands differed among materials. Band 255 was visualized in 88% of the bottles analysed, however, this band was very faint in glass bottled-water samples was very faint and was associated to an uncultured bacterium. Finally, band 243 was detected in 69% of the samples with low intensity, and it was associated to *Schlegelella*.

In brand C, PET-and glass-bottled water showed also similar fingerprints one day after bottling (Fig. 2E and 2F) (61% Dice similarity coefficient). While DGGE fingerprints remained constant in glass–bottled water for 15 days, in PET–bottled water samples, the DGGE fingerprint had

already changed within a week after bottling although remained stable afterwards (Fig. 3C). The number of bands was rather constant for C PET bottle samples, between 7 and 8 bands were visualized in the DGGE patterns. The DGGE fingerprints of PET-bottled water were stable over the 3 months studied, they clustered very tightly together, since 3 bands were present throughout the study. Band 480, 476 and 473 were present in all the C PET samples indicating that certain species persisted in mineral water. These bands belonged to *Hydrogenophaga palleronii* and *Acidovorax radices* respectively, whereas band 473 was related to an uncultured bacterium from the microbiota associated to a biodegradation process in soil (Table 1). Band 281 acquired importance in the samples after long-term storage and it was associated to an *Alphaproteobacteria*.

More bands were detected in fingerprints of C glass-bottled water (14 bands in average) than in fingerprints of C PET-bottled water (8 bands in average). Contrary to what happened in C PET-bottled water, there were greater variations of band patterns over time in glass-bottled water. Fingerprints of C glass-bottled water were constantly changing (Fig. 2F). Although the same bands were detected in the different samples, the intensity of them changed. Band 476 was shared by all glass-bottled samples. Band 436 appeared 7 days after bottling, whereas band 181 appeared 15 days after bottling. Although these bands were not detected after bottling; they could be detected in all the samples afterwards. It was not possible to assign a genus to band 181 since three different species showed 100% similarity. Band 436 was associated to an uncultured bacterium.

A total of 4 bands (281, 431, 476, 480) were observed in common in C PET- and C glass-bottled samples. Band 476 was detected in all the brand C samples. This band was prominent in PET-bottled water while the intensity of this band increased over time in glass-bottled water. Band 281, 431 and 480 were present in 13 out of 16 samples.

Taking all the samples together (different brands and materials), the cluster analysis of DGGE fingerprints for the whole samples (Fig. 4) revealed a tendency of clustering by bottle material and origin, supporting the information obtained with the clustering for each brand individually. Only samples after 24 hours after bottling clustered more closely, thereafter each brand and material evolved in a particular way. The communities from different brands did not tend to converge after long-term storage (90 days), but preserved their own identity. Nevertheless, glass-bottled waters from brand C were grouped into three different clusters depending on the

days in storage. Moreover, water 60 days after bottling from PET-bottled water clustered separately from the PET-bottled waters in brand B.

Jackknife analysis indicated that not all the mineral waters had the same internal accuracy for fingerprint classification (Table 2). Higher rates of internal accuracy were observed for brand A as compared with those from brand B and C. A PET-bottled water fingerprints were always correctly classified (100%), while A-glass bottled water fingerprints often were misclassified as A-PET bottled water. Eighty-eight percent of the samples were correctly classified in A-glass, B-glass and C-PET bottled water fingerprints. C glass-bottled water fingerprints were most often misclassified as A PET-, B glass-, C PET- bottled water (12, 12, 13%, respectively). As noted, 50% of DGGE fingerprints of B-PET bottled water were identified as A glass-bottled water.

Multivariate analysis of the DGGE fingerprints revealed differences among mineral water brands (Fig. 5). Axis x explained 31.6% of the bacterial community variation. Axis y explained 15.9% of the variation and axis z 11.7%. The set of the three axes represented a 59.3% of the variation. This analysis demonstrated the separation of the bacterial communities depending on the brand and bottle material. Brand A PET-bottled, brand A glass-bottles and brand C PET-bottled were clearly separated from the other brands. The distribution of B (PET- and glass-bottled) and C glass-bottled were close, reflecting the low variation observed between these mineral water brands.

Diversity analysis

The Shannon diversity patterns overtime were very similar for all the mineral water brands tested (Fig. 6). The diversity was higher after bottling and decreased after day 15. From this point, the diversity indices remained constant. As has been shown in the number of DGGE bands, B PET-bottled water and C-glass bottled water had a higher diversity than their respective water bottles. Whereas, in mineral water A, the diversity was similar in both material; PET- and glass bottled and did not decrease during the period analysed. In this mineral water (A) the number of DGGE bands were constant overtime.

DGGE analysis of bottles from different batch

In general, the DGGE fingerprints from bottles randomly selected and purchased in a local retailer from a different batch were different in comparison to the DGGE fingerprints more closely related with regard to the number of days after bottling to the batch analysed during 3 months (DGGE fingerprints not shown). The Dice coefficient similarities between the DGGE fingerprints of bottled waters selected randomly and the DGGE fingerprints of the batch studied were lower between 36-56%. Nevertheless, some bands from the randomly bottle water samples matched with those previously detected (Table 3).

Discussion

Three different mineral water brands were selected to study changes on the bacterial communities present in mineral waters bottled in two commonly used materials, PET and glass.

The total bacterial counts one day after bottling were about $1x10^3$ cell/ml in all the water brands analysed, but more than 90% were damaged cells. Thereafter, between one and two weeks after bottling, the total number of cells increased up to 1-2 $\log_{10}$ units. This is in accordance with previous studies which concluded that the total cell counts were constant during the first months after bottling after reaching a value of about $1x10^4$ cell/ml - $1x10^5$ cell/ml (Defives et al., 1999; Loy et al., 2005).

The viable counts accounted for less than 10% of the total cells counts immediately after bottling. Nevertheless, the community in mineral water bottles experienced a rapid transition from predominantly inactive cells to actively cells a few days after bottling. The highest increase in the viable counts occurred in the samples from glass bottles at days 15 or 21, reaching values higher than their respective water brand bottled in PET. The increase in glass-bottled waters was one week later than in PET bottled waters indeed. The increase after bottling may be due to the lysate of dead cells produced during the bottling process which could have provided nutrients to support this growth or due to an adaptation of the community to an enclosed oligotrophic environment.

No culturable bacteria were detected in brand B and low counts were obtained for both waters A and C after bottling. The initial increase in the culturable counts occurred before in glass bottles samples. After the initial increase, culturable bacteria counts fluctuated over time, in general, periods of growth followed by declines were observed within the period studied without a tendency. The culturable counts obtained were higher in glass-bottled water from brands A and

B, than in PET-bottled water, whereas brand C showed higher counts in PET-bottled water. Nevertheless, the culturable counts represented less than 10% of the total cell counts in the majority of samples analysed. Therefore, the fluctuations in the number of culturable cells were not reflected in DGGE fingerprints. It has been considered that, based on the 16S rRNA gene sequences, DGGE allows detection of bacteria that constitute up to 10% of the bacterial community (Muyzer and Smalla, 1998). A previous study concluded that bacterial retrieved by cultivation methods did not constitute the dominant populations of the whole community (Burtscher et al., 2009). In any case, the bottle material did not affect the total, viable and culturable counts after the initial increase observed between two weeks after bottling.

The DGGE fingerprints obtained with the universal primers V3 were unique for each brand. The temporal variations in the DGGE fingerprints were confirmed by analysis of the similarity among samples of digitized DGGE fingerprints. The diversity appeared greatest on samples at day 1 and 7 after bottling since more bands were detected. The communities changed upon bottling, reflected by the decrease in the number of bands and the increase in the dominance of certain bands.

The DGGE fingerprints of brand A glass-bottled water, brand B glass-bottled water and brand C PET-bottled water were different between one day after bottling and those obtained one week after bottling. During this week, the total cell counts in these waters increased more than their respective counterparts (A PET-bottled water, B PET-bottled water and C glass-bottled water), therefore the increase of total counts could have led to DGGE fingerprints changes. The microbial communities of the water brands in which the DGGE fingerprints changed during the first week, remained constant afterwards as deduced from their DGGE fingerprints. In these brands a rapid adaptation of the microbial community could have occurred. In contrast, the water brands that the DGGE fingerprint differentiated later than the initial (B PET-bottled water and C glass-bottled water), the DGGE fingerprints changed in terms of band number, position and intensity over the 3 months, with the exception of A PET-bottled water. The DGGE fingerprint of the latter remained constant over the 3 months. In addition, the bands that remained constant during the three months were different for the same water bottled in different material.

The cluster analysis of all the DGGE fingerprints revealed that the samples clustered mainly accordingly to their origin and material, in accordance to previous studies in which it is described that each mineral water spring has a particular microbial community (Loy et al., 2005; Rosenberg, 2003). The DGGE fingerprint signatures appeared to be brand and material specific, although some common bands could be observed across different brands. Furthermore, the

mineral water maintained a characteristic molecular signature even in long-term storage since the DGGE fingerprints did not converge. For this reason, and as shown in PCA analysis, it could be possible to differentiate mineral water according to their DGGE fingerprints. Although the DGGE fingerprints obtained from another batch showed lower Dice similarities than their corresponding sample, some of the most frequently observed bands were also present. Different storage conditions of these bottles could have led to a different microbial community, since then were obtained from a retailer. In consequence, it could be possible to select certain bands so as to differentiate among brands and use these bands as a microbial marker.

In conclusion, no difference in the number of total, viable and culturable bacteria counts were observed among mineral water bottled with PET and glass during long term storage. Nevertheless, the bottle material may have affected the community structure development since the DGGE bands detected in all the samples from the same brand were different for PET and glass bottles and the bottles were filled in the same day. Some of the water brands and/or material had a distinct microbial community structure clearly distinguishable from the others, which suggests the possibility of defining a molecular marker for traceability.

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Tables and figures

Table 1. Frequency of detection and identity with the closest relatives of the DGGE bands. Frequency*: number of samples with the band detected/ total number of samples of the batch study.

Mineral water sample	Band name	Frequency *	Closest relative (% sequence similarity) and its accession no.	
A PET, A glass	219	8/8, 2/8	*Hyphomicrobium* sp. (99%)	LN876552.1
A PET, A glass, C PET, C glass	480	8/8, 4/8, 8/8, 5/8	*Hydrogenophaga palleronii* (100%)	NR_114132.1
A PET, A glass	292	8/8, 8/8	*Rhodococcus* sp. (99%)	KX064755.1
A glass	271	8/8	*Acinetobacter johnsonii* (100%)	NR_117624.1
A PET, A glass, C PET, C glass	281	3/8, 8/8, 7/8, 6/8	*Novosphingobium* sp. (100%) *Sphingomonas* sp. (100%)	LC133664.1 KJ654775.1
A PET, A glass, B PET	241	8/8, 3/8, 6/8	Uncultured bacterium (100%)	KT714231.1
B PET, B glass	255	6/8, 8/8	Uncultured bacterium (100%)	GU742462.1
B PET, B glass	155	6/8, 4/8	*Porphyrobacter sanguineus* (100%)	NR_113808.1
B PET	254	6/8	*Leptospira* sp. (99%)	KX245334.1
B PET, B glass	170	3/8, 6/8	*Ramlibacter henchirensis* (99%)	NR_025203.1
B PET, B glass	243	5/8, 6/8	Uncultured *Schlegelella* sp. (99%)	GQ243114.1
B PET, C PET, C glass	253	6/8, 1/8, 2/8	*Leptospira* sp. (100%)	NR_044042.1
B PET, B glass, C PET, C glass	476	2/8, 8/8, 8/8, 8/8	*Acidovorax radicis* (99%)	NR_117776.1
C PET, C glass	473	8/8, 1/8	Uncultured bacterium (100%)	KX670409.1
C glass	436	7/8	Uncultured bacterium (94%)	LC023390.1
B glass, C glass	181	6/8, 6/8	*Oligotropha carboxidovorans* (100%) *Rhodopseudomonas pseudopalustris* (100%) *Afipia massiliensis* (100%)	NR_074142.1 NR_036771.1 NR_122099.1

Table 2. Jackknife analysis results. Numbers represent the percentage of DGGE fingerprints of bottled water assigned to each brand and material group. The number of misidentifications for members of each group is given in the columns. Note that the values in the matrix are not reciprocal, the matrix is not symmetric.

	A PET	A glass	B PET	B glass	C PET	C glass
A PET	100	12	12			12
A glass		88	50	12	12	
B PET			25			
B glass				88		12
C PET					88	13
C glass			13			63
	100	100	100	100	100	100

Table 3. Dice similarity of DGGE fingerprints between the randomly bottled waters and the closest DGGE fingerprint of the batch study. Number of the common bands observed between the DGGE fingerprints of bottles randomly selected and the DGGE fingerprints of the batch study. Frequency*: number of samples with the band detected/ total number of samples of the batch study.

Mineral water sample randomly selected (days after bottling)	Dice similarity coefficient (%)	Mineral water sample	Common bands	
			Band name	Frequency*
A PET R (9)	56	A PET 7	219	8/8
			216	7/8
			269	6/8
A glass R (62)	36	A glass 60	292	8/8
			269	7/8
B PET R (21)	48	B PET 21	253	6/8
			243	5/8
B glass R (78)	40	B glass 90	476	8/8
			181	6/8
C PET R (30)	50	C PET 30	480	8/8
C glass R (11)	38	C glass 15	431	6/8

Figure 1. Changes in the number of total and viable cells and culturable counts in mineral water (A, B and C) bottled in PET and glass.

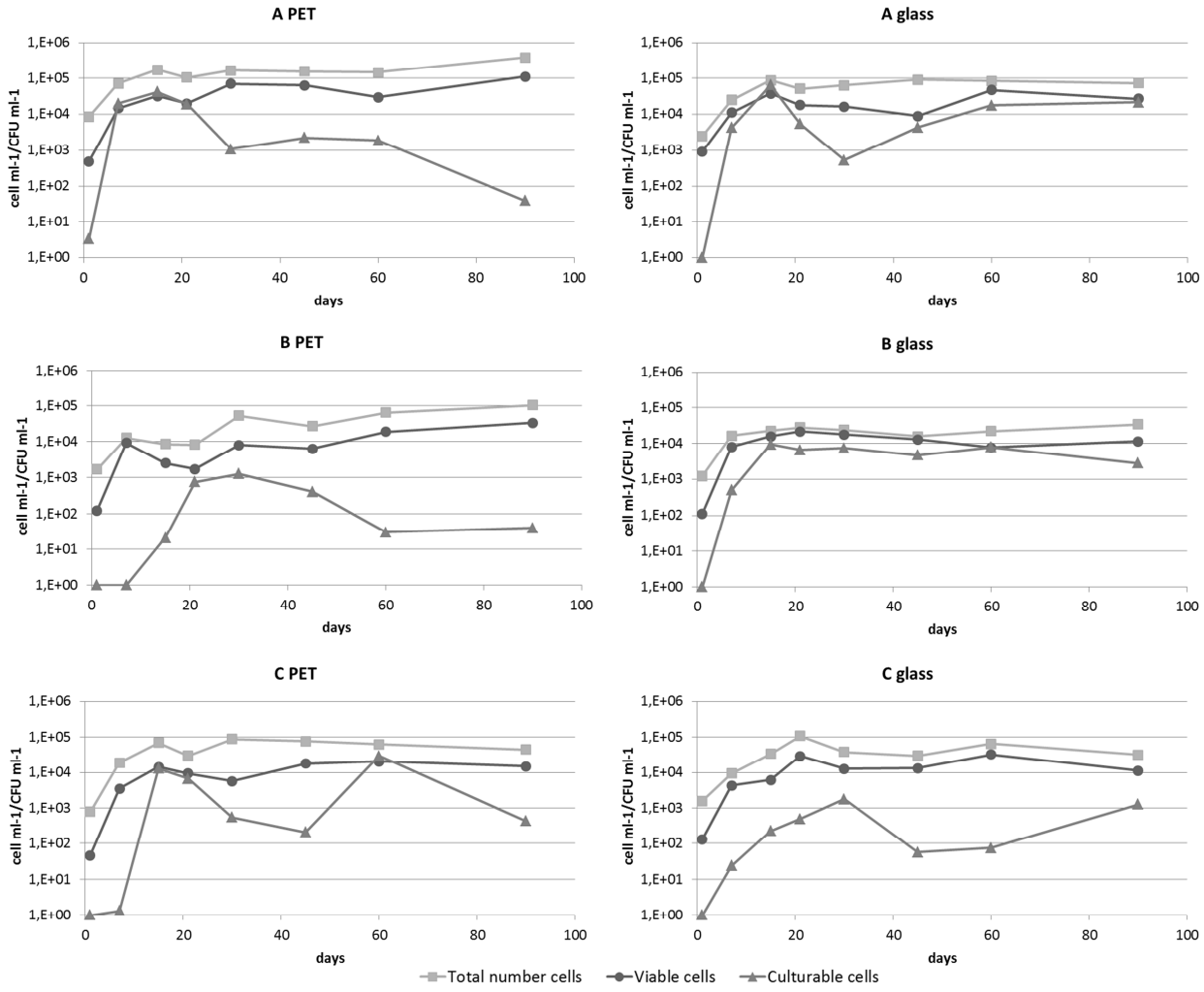


Figure 2. DGGE fingerprints of the 3 water mineral water brands analysed at 1, 7, 15, 21, 30, 45, 60 and 90 days after bottling, bottled in PET and glass bottles. Identified bands are given numbers.

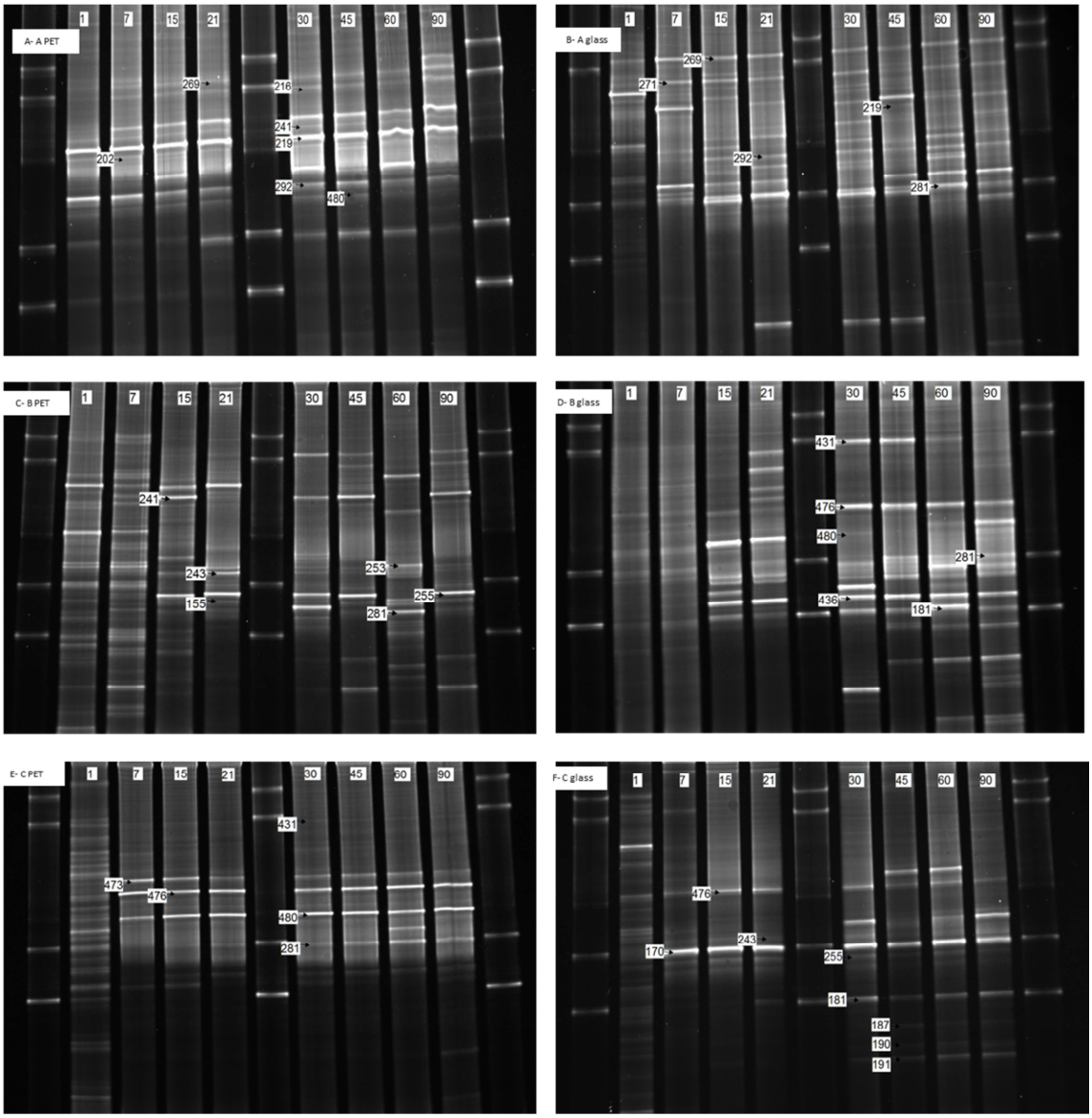


Figure 3. Cluster analysis of DGGE fingerprints with similarity matrix for (a) A PET and A glass mineral water, (b) B PET and B glass mineral water and (c) C PET and C glass mineral water. Clustering was performed using Dice's coefficient and UPGMA.

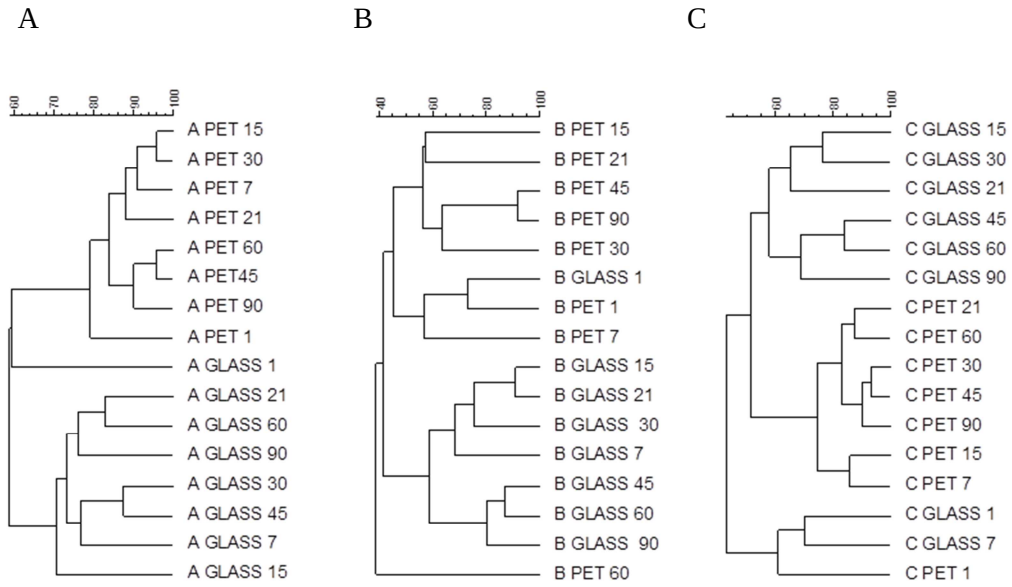


Figure 4. Cluster analysis of all the mineral water brands using Dice's coefficient and UPGMA.

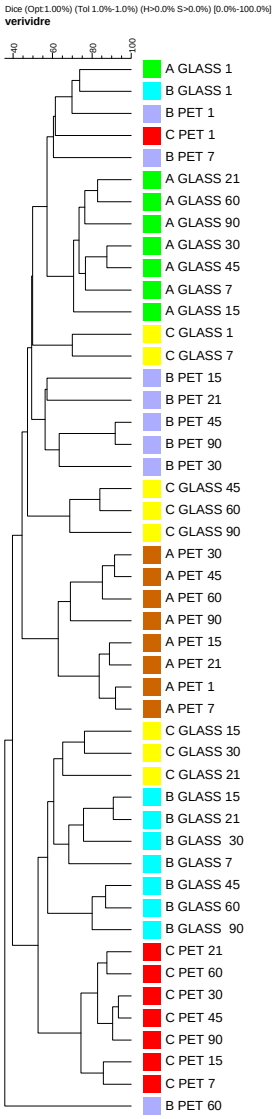


Figure 5. Principal components analysis (PCA) with the DGGE fingerprints. PC1, PC2 and PC3 represented in x, y, z axes, respectively.

	A PET		A glass		B PET		B glass		C PET		C glass

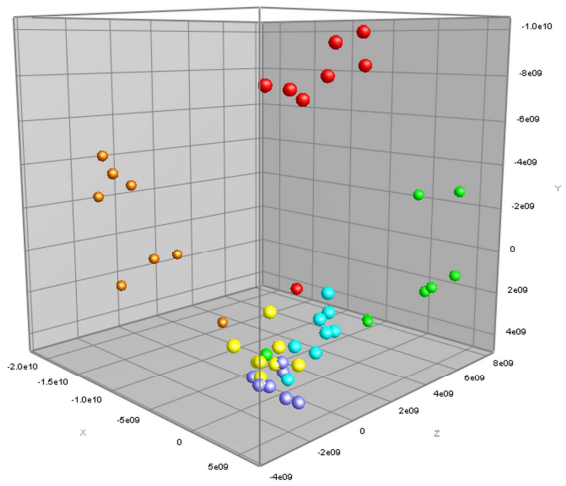
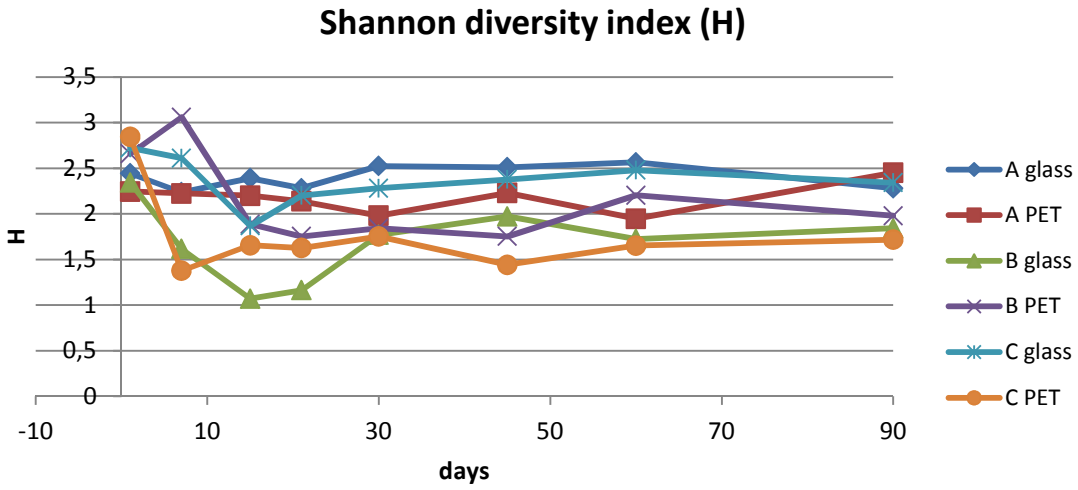


Figure 6. Changes in the Shannon-Weaver index of diversity based on the number and relative intensities of the bands of the DGGE profiles.



4.3. Article 3

Use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry for bacterial monitoring in routine analysis at a drinking water treatment plant

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Les estacions de tractament d'aigua potable (ETAP) utilitzen diversos tractaments per a garantir la salubritat de l'aigua pel consum humà que es distribuirà a través de la xarxa, segons regula la Directiva Europea 98/83/CE. El coneixement de les comunitats microbianes de l'aigua pot millorar la gestió d'aquestes ETAPs, no obstant això, es necessari desenvolupar noves tècniques analítiques suficientment eficients, robustes, ràpides i senzilles per a la seva aplicació en l'anàlisi de rutina dels laboratoris de control de l'aigua. Les tècniques basades en tests fenotípics, per exemple les galeries API® (BioMérieux) s'utilitzen freqüentment per a la identificació dels microorganismes aïllats en els laboratoris de rutina. Encara que, existeixen una sèrie de tècniques per a l'estudi de les comunitats bacterianes d'ambients complexos com el sistema PhenePlate™ (PhPlate Microplate Techniques AB) juntament amb la seqüenciació del gen ARNr 16S, amplificació aleatòria d'ADN polimòrfic (RAPD), anàlisi multilocus de les seqüències (MLSA), electroforesi en gel de camp polsat (PFGE), electroforesi en gel amb gradient desnaturalitzant (DGGE), la seqüenciació massiva i la hibridació *in situ*, aquestes tècniques no són aplicables en anàlisis de rutina per la seva complexitat. Darrerament, entre les metodologies d'identificació ràpides, l'espectrometria de masses de desorció/ionització amb làser assistida per matriu acoblada amb un analitzador de tems de vol (MALDI-TOF MS) ha emergit com una tècnica amb grans potencials i ha estat implantada satisfactòriament en el camp del diagnosi clínic, però pocs estudis han validat aquesta tècnica per a soques ambientals.

En aquest estudi, es va avaluar l'ús d'aquesta tecnologia emergent, MALDI-TOF MS, per a l'anàlisi de rutina en una ETAP. Per aquest motiu, es va comparar la identificació de soques aïllades en una planta potabilitzadora mitjançant la tècnica MALDI-TOF MS amb el sistema PhenePlate™ juntament amb la seqüenciació del gen ARNr 16S i les galeries API 20NE (BioMérieux).

Les soques aïllades de l'aigua en l'ETAP de Sant Joan Despí van ser identificades mitjançant MALDI-TOF MS (Bruker Daltonics GmbH) amb el mètode de transferència directa. Paral·lelament, es va realitzar un fenotipatge bioquímic de les soques utilitzant les microplaques PhP-48 del sistema PhenePlate™. A partir dels grups fenotípics obtinguts, es va seleccionar la soca representant de cada grup i es va procedir a seqüenciar el gen del ARNr 16S. Paral·lelament, les soques representants es van identificar amb les galeries API 20NE. Els valors que es van prendre de referència per a la identificació a nivell d'espècies van ser els següents: obtenció d'una puntuació superior a 2,000 per MALDI-TOF MS, una similitud del 97% pel gen ARNr 16S i per API una identificació superior al 90% ID.

Un total de 277 soques van ser aïllades i identificades, un 26% de les soques es van poder identificar a nivell d'espècie mitjançant la seqüenciació del gen ARNr 16S amb un similitud superior al 97%. Les limitacions de la identificació a partir del gen ARNr 16S a nivell d'espècie són conegudes, per tant, la seqüenciació conjunta amb altres gens conservats milloraria la identificació. Les galeries API® van permetre identificar a nivell d'espècie un 43% de les soques, mentre que el MALDI-TOF MS un 39%. API i MALDI-TOF MS van obtenir uns percentatges d'identificació molt similars, tot i que el nombre de soques que es va caracteritzar mitjançant la galeria API va ser menor. El 80% de les soques van obtenir el mateix resultat a la identificació comparant els resultats obtinguts entre les tècniques MALDI-TOF MS i la seqüenciació del gen ARNr 16S. Mentre que els resultats entre MALDI-TOF MS i API van obtenir un 75% de coincidència. Respecte la seqüenciació del gen ARNr 16S i API, el valor va ser de 50%. Les discrepàncies en les identificacions entre el gen ARNr 16S amb les metodologies MALDI-TOF MS i API van ser degudes principalment a una falta de dades de les biblioteques disponibles d'aquestes dues. Els agrupaments de les soques per similitud entre els espectres proteics del MALDI-TOF MS i els perfils bioquímics, tot i ser dades de naturalesa diferent, van ser semblants, indicant una resolució taxonòmica similar per a les dues tècniques.

La utilització de MALDI-TOF MS va ser comparable amb el sistema PhenePlate™ (80%). Així doncs, la identificació mitjançant MALDI-TOF MS és una metodologia útil i prometedora en laboratoris de rutina d'anàlisi d'aigua de consum, gràcies a la seva robustesa i rapidesa per a la identificació bacteriana. No obstant això, els espectres proteics de soques ambientals existents a les bases de dades són limitats. En conseqüència, es requereix una ampliació de les bases de dades amb soques ambientals per a poder aconseguir una correcta identificació de les soques aïllades a l'aigua.

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Use of matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry for bacterial monitoring in routine analysis at a drinking water treatment plant

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ABSTRACT

The study of bacterial communities throughout a drinking water treatment plant could provide a basic understanding of the effects of water processing that could then be used to improve the management of such plants. However, it is necessary to develop new analytical techniques that are sufficiently efficient, robust and fast for their effective and useful application in routine analysis. The aim of this study is therefore to assess the performance of matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS), as compared to the PhenePlate™ system, for routine analysis in a drinking water treatment plant. To this end we studied a total of 277 colonies isolated in different seasons and from different points throughout the water treatment process, including: raw water, sand filtration, ultrafiltration, reverse osmosis and chlorination. The colonies were analysed using MALDI–TOF MS by direct deposition of the cells on the plate. The colonies were also biochemically fingerprinted using the PhenePlate™ system, clustered according to their similarity and a representative strain was selected for 16S rRNA gene sequencing and API® gallery-based identification. The use of MALDI–TOF MS was reliable compared to the PhenePlate™ system and has the advantage of being faster and relatively cheap. Bacteria typing by MALDI–TOF MS is therefore a promising method to replace conventional routine phenotypic methods for the identification of bacteria in drinking water laboratories, thanks to its robustness. The major limiting factor for MALDI–TOF MS is the lack of a suitable mass spectra database; although each laboratory can develop its own library. This methodology will provide a tracking tool for companies to use in risk management and the detection of possible failures in both the water treatment processes and the distribution network, as well as offering characterization of the intrinsic microbial populations.

Introduction

Drinking water treatment plants apply different treatments, depending on the quality of the source water, to achieve adequate chemical and microbiological quality in accordance with the European Directive 98/83/EC. The Directive sets out the essential quality standards and defines the microbiological indicators that must be monitored in drinking water and tested regularly (Anonymous, 1998).

In a standard drinking water microbiology laboratory, the current methods for assessing the impact that water may have on the health of consumers are mainly based on classical culture-dependent methods: well-established approaches for the detection and enumeration of bacterial indicators and pathogens (Casanovas-Massana and Blanch, 2013; Vilanova et al., 2004). Nevertheless, drinking water and the systems used to distribute it contain a wide range of microorganisms, which are representative of different ecosystems (Eichler et al., 2006; Poitelon et al., 2010, 2009; Revetta et al., 2010). A large portion of this microbial community is still unexplored as are its role within the ecosystem and the physiological impact of the different microorganisms (Pinto et al., 2012). Therefore, knowledge of the microbial community could contribute to improving water management policies.

In standard water plant laboratories, the techniques most frequently used to identify microorganisms are based on conventional phenotypic tests. Among the rapid typing methods that have been developed, the API® system (BioMérieux, France) is one of the most frequently used biochemical typing method. However, previous

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studies use this method to characterize isolates from drinking water (Kühn et al., 1997; Penna et al., 2002; Ribas et al., 2000).

Another typing method used to study bacterial communities is the PhenePlate™ system (PhP system) (PhPlate Microplate Techniques AB, Sweden): a miniaturized biochemical phenotyping method based on evaluation of the kinetics of several biochemical reactions performed in microtiter plates. The PhP system has been shown to be useful in typing different groups of microorganisms, such as faecal coliforms and enterococci populations in sewage wastewater (Vilanova and Blanch, 2005; Vilanova et al., 2004), the microorganisms found in natural swimming pools (Casanovas-Massana and Blanch, 2013), heterotrophic microbial populations in mineral waters (Casanovas-Massana and Blanch, 2012) and *Pseudomonas aeruginosa* (Casanovas-Massana et al., 2010). After typing the isolates, cluster analysis can be performed and a strain that is representative of the cluster can be identified, thereby reducing the number of isolates on which to perform the final identification test, for instance through 16S rRNA gene sequencing. Furthermore, many studies have used the 16S rRNA sequencing gene to characterize the microbial populations in drinking water plants or networks (Poitelon et al., 2010; Revetta et al., 2010).

Characterization of these diverse and variable microbial communities by traditional phenotypic and genotypic methods, such as conventional biochemical testing, API, the PhP system, DGGE, in situ hybridization, RAPD, MLSA and PFGE, is a prohibitively costly activity (Dieckmann et al., 2005; Welker, 2011). Therefore, there is a need to develop and implement new efficient and fast analytical techniques which could be adopted by standard drinking water laboratories. The information on microbial communities in drinking water could then be used to develop tracking tools to rapidly detect possible failures in the network and moreover, to identify potential pathogens that may pose a public health risk (Szewzyk et al., 2000).

In recent years, different high-throughput screening technologies have been developed which can provide large amounts of data from a single experimental run. For instance, next generation sequencing, which is currently being used in different studies (França et al., 2014; Pinto et al., 2012; Vaz-Moreira et al., 2011), has been proposed for microbial identification and characterization based on sequencing of certain genes, such as 16S rRNA. However, this technology is not suitable for routine analysis due to its cost and its technical complexity (Welker, 2011). Recently, mass spectrometry-based proteomics using matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF MS) has emerged as a promising and reliable tool for bacteria identification, especially in clinical diagnostic microbiology (Clark et al., 2013; Croxatto et al., 2012; Sogawa et al., 2011; Welker and Moore, 2011) and for environmental strains (Ruelle et al., 2004; Welker and Moore, 2011). Many studies have successfully used MALDI–TOF MS to identify human pathogens isolated from clinical samples (Alatoom et al., 2011; Barberis et al., 2014; Calderaro et al., 2014; Hsieh et al., 2008; Seng et al., 2010; Vila et al., 2012) and it has been proposed as a replacement for conventional phenotypic identification (Bizzini and Greub, 2010; Carbonnelle et al., 2011; Croxatto et al., 2012; Saffert et al., 2011; Seng et al., 2009). However, there is little knowledge regarding the applicability of this method for the identification and monitoring of environmental bacteria. To the best of our knowledge, only a few studies have applied this method to analyse bacterial isolates from hypersaline sediments (Munoz et al., 2011), spinach processing plant (Hausdorf et al., 2013), the rhizosphere of plants and soil (Ghyselinck et al., 2013, 2011; Uhlik et al., 2011), *Aeromonas* strains from environmental samples (Benagli et al., 2012), bacteria from marine sponges (Dieckmann et al., 2005) and ballast water (Emami et al., 2012).

The aim of this work was therefore to assess the feasibility and performance of MALDI–TOF MS, as compared to the PhP system, as a means to characterize and identify a collection of strains isolated from a drinking water treatment plant during routine analysis.

Materials and methods

Sampling

Samples were collected from a drinking water treatment plant in Sant Joan Despí (Barcelona). Water is pumped from a river and pre-treated by sequential treatments. Primary treatment includes addition of ClO_2 to oxidize organic matter, iron and manganese and disinfect. Subsequently, a coagulation and flocculation step followed by a sedimentation and decantation step (to eliminate the flocculants) are performed. Finally, a sand filtration step removes suspended solids from the water. At that point, the pre-treated water is mixed with groundwater, which is collected via wells from the aquifer. An ultrafiltration step is performed so as to meet the requirements for reverse osmosis. Subsequently, the water is processed by reverse osmosis; and finally, remineralization and chlorination steps are applied. The resultant disinfected water then enters the distribution system.

The samples were gathered at 5 different stages of the water treatment: raw surface water (R), after sand filtration (S), after the membrane ultrafiltration (U), after reverse osmosis (O) and from the drinking water reservoir after chlorination (T). The samples were collected during two different seasons. The first sampling campaign was carried out during winter, when the temperature of the water was at its annual lowest, around 13 °C; while the second was performed during summer, when the temperature of the water was at its highest, 22 °C.

Volumes of between 0.001 ml and 1 l were filtered through 0.45 μm polycarbonate membranes within 3 h of collection. The filters were incubated on Water Plate Count Agar plates (Oxoid, United Kingdom) at 22 °C ± 2 °C for 3 days. After incubation, a maximum of 30 isolates from each step were randomly selected from the plates and subcultured on the same medium. This medium is suitable for the cultivation of microorganisms found in water, in accordance with ISO 6222:1999 (Anonymous, 1999).

Biochemical fingerprinting: The PhenePlate™ system, PhP-48

The isolates were biochemically phenotyped using the PhenePlate™ system (PhP system, PhPlate Microplate Techniques AB, Sweden), using PhP-48 plates, consisting of 48 different dehydrated reagents selected to provide optimal discrimination and reproducibility for the bacterial group studied (Inger, 1985; Kühn and Möllby, 1993; Kühn et al., 1997). The assay was performed according to the manufacturer's instructions. The cultures were prepared on Water Plate Count Agar plates for 72 h ± 2 h at 22 °C ± 2 °C. Cell suspensions were prepared by resuspension of the cultures in a distilled water solution of 0.1% w/v proteose peptone (Difco, United States) and 0.011% w/v bromothymol blue. Aliquots of 150 μl were transferred into each well, and they were incubated at 22 °C ± 2 °C. Absorbance values for reactions were read after 16 h, 40 h, 64 h, 88 h and 122 h at 620 nm with a spectrophotometer: iEMS Reader MF (Labsystems, Finland). *P. aeruginosa* NCTC 10332$^{(T)}$ was used as an internal control in all the experiments to confirm reproducibility. The biochemical profiles were calculated using cumulative absorbance values, as previously described (Kühn et al., 1991), using the PhPWin® (PhPlate Microplate Techniques AB, Sweden) software.

The biochemical fingerprints of all the isolates were compared pairwise; the correlation coefficient was calculated and used to represent the similarity between each pair. The similarity matrix thus obtained was subjected to cluster analysis using the unweighted-pair group method with arithmetic mean (UPGMA). Isolates with

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a correlation coefficient ≥0.950 were regarded as belonging to the same PhP system type, or cluster. The isolates showing the highest mean similarity within a cluster and the lowest correlation with other clusters were selected as the representative strains (Kühn et al., 1991) of phenotypic clusters for further identification. A representative strain from each phenotypic cluster with 2 or more isolates was selected for further analysis, including API identification and 16S rRNA gene sequencing.

Genotypic characterization

As stated above, the representative strain from each PhP system cluster with 2 or more isolates was selected for genotypic characterization. The strains were cultured on Tryptic Soy Broth (TSB) (Pronadisa, Spain) at 22 °C ± 2 °C for 24 h ± 2 h. DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Spain) following the manufacturer's instructions. Partial amplification of the 16S rRNA gene was performed using the universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′), as previously described (Casanovas-Massana et al., 2010). Nucleotide sequences were checked for their quality and compared with the sequences deposited in the 16S ribosomal RNA sequences (Bacteria and Archea) database of the National Center for Biotechnology Information using BLAST. Amplified sequences were deposited in GenBank under the accession numbers shown in Table A1. A cut-off at ≥97% similarity was used for strain identification at the species level (Stackebrandt and Goebel, 1994).

API galleries

The representative strain from each PhP system cluster was identified using the API 50CHB or API 20NE system, according to the Gram stain, following the manufacturer's instructions (BioMérieux, France), since all the isolates were oxidase positive. The API profiles obtained were identified using the APIWEB™ database V8.0. A 90% identification accuracy (90%ID) was used as a threshold for reliable identification at the species level, in accordance with the manufacturer's instructions. When the 90%ID level was not met, the isolate was classified with "low probability".

Mass spectrometry analysis

The isolates were analysed by MALDI–TOF MS (Bruker Daltonik GmbH, Germany) as previously described (Vila et al., 2012). Briefly, each isolate was incubated overnight on Water Plate Count Agar and a small amount of the resultant growth was extended over a metallic MALDI–TOF 96 plate, which was subsequently covered with 1 μl of matrix (saturated alpha-cian-4-hydroxycinamic acid–50% acetonitrile–2.5% trifluoroacetic acid). Then, the sample was allowed to air dry for 5 min before testing. MALDI–TOF MS was performed with a MicroFlex II mass spectrometer (Bruker Daltonik GmbH, Germany) equipped with a 60 Hz laser, according to the manufacturer's recommendations. The spectra obtained were analysed using Bruker MALDI Biotyper v2.0 software (Bruker Daltonik GmbH, Germany). The similarity of the patterns to the representative species in the database was represented as a score. The results were categorized as follows: score <1.700, no reliable identification; score between 1.700 and 1.999, identification at genus level; and score ≥2.000, species identification. Control strains (*Acidovorax delafieldii* CECT 4105 and *Pseudomonas putida* CECT 324) were correctly identified at the species level using MALDI–TOF: they obtained scores ≥2.000.

The mass spectra were converted to txt files and were imported into BioNumerics 7.5 software (Applied Maths, Belgium). Before clustering, the spectra were subjected to baseline subtraction with a threshold for peak acceptance signal-to-noise ratio of 10. The similarity between the spectra was analysed using a peak-based Pearson correlation and the spectra were clustered using the UPGMA clustering algorithm.

Table 1
Origin and seasonal distribution of the isolates used in the study.

Step	No. of isolates in winter	No. of isolates in summer
Raw water (R)	26	30
After sand filtration (S)	24	25
After ultrafiltration (U)	25	29
After reverse osmosis (O)	29	30
After chlorination treatment (T)	29	30

Results and discussion

A total of 277 colonies were isolated from the whole of the water treatment process (133 in winter and 144 in summer), as shown in Table 1. Of these, 100 isolates showed unique PhP system phenotypes (single strains); while a further 57 isolates presented only a weak reaction in the PhP system plates and were not included in the study. The remaining 120 isolates, out of 277, were grouped into 28 phenotypic clusters, each with a correlation coefficient higher than 0.950, according to the PhP system. All the strains belonging to the same PhP system cluster were phenotypically identical, so a representative strain from each of the 28 groups was selected and its 16S rRNA gene was sequenced. The species assigned by 16S rRNA sequencing was extrapolated to all the strains in the cluster, in accordance with the manufacturer's instructions.

The 16S rRNA gene sequencing allowed identification of all the isolates at the genus level (Table 2); although we were only able to identify 26% of the isolates (31) unequivocally at the species level, with a similarity ≥97%, with respect to the sequences available in the GenBank database. Thus, a total of 89 isolates (74%) could not be unambiguously assigned to a single species, since the sequences were 99% identical to more than one species belonging to the same genus. Therefore, these isolates were only identified at the genus level. The 16S rRNA gene sequencing results identified a total of 14 genera (Table A2); the highest proportions of the isolates were affiliated to *Acidovorax* sp, *Chryseobacterium* sp., *Cloacibacterium* sp. and *Pseudomonas* sp. The sequences that were identified at the species level belonged to *A. delafieldii*, *Acidovorax temperans*, *Bacillus cereus*, *Bacillus thuringiensis*, *Cloacibacterium normanense*, *Flavobacterium oncorhynchi* and *Ralstonia pickettii*. Sequencing of housekeeping genes together could improve the identification of various groups of microorganisms at the species level, as suggested by (Ng et al., 2014).

The representative strains were also identified using the API rapid biochemical typing method (Table 2). A total of 12 strains out of the 28 (43%) were assigned by the API system at the species level with a high probability (90%ID); while the other 16 strains (57%) were not identified due to the low probability obtained (below the 90%ID threshold). It has been reported that the accuracy of identification by API galleries strongly relies upon the robustness of the database (Bosshard et al., 2006). For environmental isolates, the conditions for the use of this test are too stringent and make it

Table 2
Percentages of strains identified at taxonomical levels.

%	PhenePlate™ system + 16S rRNA (*n* = 120)	MALDI–TOF MS (*n* = 277)	API galleries (*n* = 28)
Species	26	39	43
Genus	74	26	
No reliable identification		35	57

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unsuitable for the expression of certain biochemical characteristics, or additional tests are required for the completion of the identification, making it difficult to identify these isolates (Croci et al., 2007; O'Hara et al., 2003; Park et al., 2011).

The MALDI–TOF MS spectra analysis (Table 2) resulted in the assignment of 39% (107 out of 277) of the isolates at the species level, similar to the value obtained with API, and 26% (73 out of 277) at genus level; 35% (97 out of 277) could not be identified to any taxon according to the database available. Thus, MALDI–TOF MS and the API galleries yielded equivalent identification percentages at the species level, although the number of strains tested by API was low to draw definitive conclusions. However, we expected MALDI–TOF MS to have a greater capacity to resolve species than API, since MALDI–TOF MS is based on a large number of variables (represented by protein differences) compared to the 20 biochemical tests provided by API. It should also be borne in mind that strain identification by MALDI–TOF MS and API was not possible for some isolates due to a lack of reference profile libraries.

A total of 60 species belonging to 25 genera were identified in water isolates, as shown in Table 3. Regarding the genus assignment by MALDI–TOF MS, the genera which were classified with high confidence at the species level were *Acinetobacter, Janthinobacterium, Micrococcus, Pseudomonas, Ralstonia* and *Shewanella*; with the majority of isolates yielding a score of ≥2.000. Meanwhile, most of the problematic identifications concerned isolates belonging to the phyla *Bacteroidetes* (*Chryseobacterium* and *Flavobacterium*) and *Firmicutes* (*Lysinibacillus*), which obtained the lowest scores and MALDI–TOF MS was incapable of identifying the isolates at the species level due to a limited database reference library; although difficulties in lysing cell wall structure have also been reported for some Gram positive rods (Barberis et al., 2014). Although 60 species were detected, 28 of the species were only represented by one isolate. The species with more than 5 strains isolated from different water treatment stages and seasons were successfully identified and belonged to *A. temperans, Acinetobacter johnsonii, Janthinobacterium lividum, Micrococcus luteus, Pseudomonas veronii* and *Shewanella baltica*. Furthermore, the *Aeromonas caviae, Bacillus subtilis* and *Elizabethkingia miricola* and isolates also showed satisfactory identification at the species level.

The results obtained by the PhP system were compared to the results obtained by MALDI–TOF MS. Only 79 out of 120 isolates that had previously been clustered by the PhP system and identified by 16S rRNA sequencing were identified by MALDI–TOF MS; so the comparison was performed using these 79 isolates. Concerning the identification of the isolates, the comparison between the two methodologies resulted in a match for 80% of the isolates. Meanwhile, all the strains successfully identified using API were compared to the results of 16S rRNA gene assignment. A total of 12 isolates were contrasted; 50% of the identifications of isolates by API matched those obtained by 16S rRNA gene sequencing. Finally, the isolates characterized by API were compared with those identified at the species or genus level by MALDI–TOF MS and 75% of them matched. Thus, high concordance was found between the isolates identified by MALDI–TOF MS and the PhP system.

As shown in Table 4, there were some discrepancies when comparing identifications by the 3 different methodologies. All the methodologies were in agreement with the identification of isolates belonging to *Chryseobacterium* sp., *Aeromonas* sp., *Bacillus* sp. and *Pseudomonas* sp.; although API failed to identify 2 isolates out of 3 belonging to *Aeromonas* sp., 1 isolate out of 2 *Bacillus* sp. and 2 isolates out of 5 *Pseudomonas* sp. Furthermore, isolates identified by 16S rRNA gene sequencing and MALDI–TOF MS as *Janthinobacterium* sp., *Microbacterium* sp., *Ralstonia* sp. and *Shewanella* sp. could not be identified by API. Meanwhile, there were differences for some isolates: API identified some strains as belonging to the *Ochrobactrum* genus whereas 16S rRNA and MALDI–TOF identified them as *Acidovorax* sp. and *Hydrogenophaga* sp. Likewise, *Flavobacterium* sp. isolates identified by 16S rRNA and MALDI–TOF were misidentified as *Sphingomonas* sp. by API; although 1 of these isolates could not be identified by MALDI–TOF. API galleries identified 3 isolates as *Brevundimonas* sp., whereas 16S rRNA gene sequencing attributed these isolates to *Bergeyella* sp. and *Cloacibacterium* sp. Finally, *Hydrogenophaga* sp., *Lysinibacillus* sp., *Bergeyella* sp. and *Cloacibacterium* sp. could not be identified by MALDI–TOF. The isolated strain of *Lysinibacillus* sp. was misidentified; perhaps because

Table 3
Number of isolates identified by MALDI–TOF MS at species (cut-off score ≥2.000) and genus (cut-off score ≤2.000) levels.

	n = 180	Identification at the species level *n* = 107	Identification at the genus level *n* = 73
Acidovorax delafieldii	13	2	11
Acidovorax temperans	6	6	
Acinetobacter baumannii	1	1	
Acinetobacter johnsonii	6	6	
Acinetobacter radioresistens	1	1	
Acinetobacter schindleri	1		1
Acinetobacter parvus	1		1
Acinetobacter towneri	1		1
Aeromonas caviae	5	4	1
Aeromonas hydrophila	4	3	1
Aeromonas jandaei	1		1
Aeromonas schubertii	2	2	
Aeromonas veronii	4	4	
Arthrobacter pigmenti	1		1
Bacillus cereus	24	14	10
Bacillus endophyticus	1	1	
Bacillus idriensis	3		3
Bacillus indicus	1	1	
Bacillus infantis	1	1	
Bacillus licheniformis	1	1	
Bacillus megaterium	2	2	
Bacillus mojavensis	1	1	
Bacillus muralis	1		1
Bacillus mycoides	2		2
Bacillus simplex	11	4	7
Bacillus subtilis	4	3	1
Bacillus thuringiensis	2		2
Chryseobacterium gleum	2	1	1
Chryseobacterium indologenes	3		3
Comamonas aquatica	1		1
Cupriavidus metallidurans	1	1	
Dickeya zeae	1		1
Elizabethkingia miricola	5	3	2
Flavobacterium hibernum	2	1	1
Flavobacterium johnsoniae	2		2
Flavobacterium saccharophilum	2		2
Janthinobacterium lividum	8	8	
Kocuria rhizophila	1	1	
Lysinibacillus fusiformis	6	1	5
Massilia sp.	1	1	
Microbacterium laevaniformans	2		2
Micrococcus luteus	5	5	
Pantoea sp.	1	1	
Pseudomonas alcaligenes	1		1
Pseudomonas anguilliseptica	3		3
Pseudomonas antarctica	1	1	
Pseudomonas fluorescens	2	1	1
Pseudomonas frederiksbergensis	2	2	
Pseudomonas gessardii	1	1	
Pseudomonas koreensis	3	1	2
Pseudomonas libanensis	2	2	
Pseudomonas synxantha	1	1	
Pseudomonas veronii	7	7	
Ralstonia mannitolilytica	3	3	
Serratia proteamaculans	1		1
Shewanella baltica	5	5	
Sphingopyxis terrae	1	1	
Staphylococcus saprophyticus	1		1
Staphylococcus epidermidis	1	1	
Wautersiella falsenii	1	1	

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Table 4
Concordances and discrepancies between 16S rRNA gene sequencing, MALDI–TOF MS and API galleries in the identification of bacteria.

16Sr RNA		MALDI–TOF MS		API Galleries	
Identification	No. of isolates	Identification	No. of isolates	Identification	No. of isolates
Chryseobacterium sp.	1	*Chryseobacterium* sp.	1	*Chryseobacterium* sp.	1
Aeromonas spp.	3	*Aeromonas* spp.	3	*Aeromonas* sp./*not identified*	1/2
Bacillus spp.	2	*Bacillus* sp.	2	*Bacillus* sp./*not identified*	1/1
Pseudomonas spp.	5	*Pseudomonas* spp.	5	*Pseudomonas* spp./*not identified*	3/2
Janthinobacterium sp.	1	*Janthinobacterium* spp.	1	*not identified*	1
Microbacterium sp.	1	*Microbacterium* spp.	1	*not identified*	1
Ralstonia sp.	1	*Ralstonia* spp.	1	*not identified*	1
Shewanella sp.	1	*Shewanella* sp.	1	*not identified*	1
Acidovorax spp.	4	*Acidovorax* sp.	4	*Ochrobactrum* sp./*not identified*	1/3
Flavobacterium spp.	2	*Flavobacterium* sp./*not identified*	1/1	*Sphingomonas* sp./*not identified*	1/1
Hydrogenophaga sp.	1	*not identified*	1	*Ochrobactrum* sp.	1
Lysinibacillus sp.	1	*not identified*	1	*not identified*	1
Bergeyella spp.	1	*not identified*	1	*Brevundimonas* sp.	1
Cloacibacterium spp.	4	*not identified*	4	*Brevundimonas* spp./*not identified*	2/2

the API database includes only 2 species of this genus. Likewise, the *Bergeyella* genus was misidentified, for which the API database also contains only 1 species. The same holds true for the *Shewanella* genus, for which only 1 reference strain is included in the API database. Noticeably, there are no entries for the *Acidovorax, Cloacibacterium, Flavobacterium, Hydrogenophaga* and *Janthinobacterium* genera in the API library supplied. It is not known whether these species could be incorporated into the API database; if they can, then identification could improve. Similarly, the misidentification of all *Bergeyella* sp., *Cloacibacterium* sp., *Hydrogenophaga* sp. and *Lysinibacillus* sp. by MALDI–TOF technology may be due to an incomplete reference data spectrum covering the genera.

As shown in Table 5, the predominant spectrum identified by MALDI–TOF MS matched with the identification by 16S rRNA gene sequencing in all the PhP system clusters; although not all the PhP system clusters contained a unique MALDI–TOF spectrum. In fact, MALDI–TOF MS assigned the same taxon to all the strains from PhP system clusters composed of *Bacillus* sp., *Ralstonia* sp. and *Shewanella* sp., according to 16S rRNA gene sequencing, without mismatches. However, clusters including representative strains of the genera *Aeromonas, Flavobacterium, Janthinobacterium, Lysinibacillus, Pseudomonas, Acidovorax, Chryseobacterium* and *Microbacterium* contained different mass spectra. None of the strains from the PhP system clusters represented by *Bergeyella, Cloacibacterium* and *Hydrogenophaga* were identified by MALDI–TOF MS.

Comparison of the dendrograms obtained from the MALDI–TOF MS and PhP system data using the Pearson correlation and UPGMA (Fig. 1) revealed that the clusters resulting from both methods were similar, although the nature of the data differs (protein profiles versus biochemical profiles). As shown in Fig. 1, the isolates were found to occupy the same position in both dendrograms, suggesting both techniques have similar taxonomic resolution. The MALDI–TOF MS dendrograms generated were in accordance with those of the biochemical analysis performed with the PhP

Table 5
Comparison of 16S rRNA gene sequencing of representative strains from PhenePlate™ system clusters with their respective MALDI–TOF MS identification.

Number of cluster PhP system profile	No. of isolates	16S rRNA identification	No. of isolates	MALDI–TOF identification
2	6	*Bacillus* spp.	6	*Bacillus* spp.
1	3	*Ralstonia* spp.	3	*Ralstonia* spp.
1	2	*Shewanella* spp.	2	*Shewanella* spp.
3	7	*Aeromonas* spp.	6	*Aeromonas* spp.
			1	*Micrococcus* sp.
2	13	*Flavobacterium* spp.	5	*Flavobacterium* spp.
			8	*no reliable identification*
1	5	*Janthinobacterium* spp.	3	*Janthinobacterium* spp.
			2	*no reliable identification*
1	3	*Lysinibacillus* spp.	1	*Lysinibacillus* sp.
			2	*no reliable identification*
5	16	*Pseudomonas* spp.	15	*Pseudomonas* spp.
			1	*Acidovorax* sp.
4	20	*Acidovorax* spp.	14	*Acidovorax* spp.
			1	*Aeromonas* sp.
			1	*Arthrobacter* sp.
			1	*Comamonas* sp.
			3	*no reliable identification*
1	22	*Chryseobacterium* spp.	5	*Chryseobacterium* spp.
			4	*Elizabethkingia* spp.
			2	*Janthinobacterium* spp.
			2	*Lysinibacillus* spp.
			1	*Bacillus* sp.
			1	*Pseudomonas* sp.
			1	*Wautersiella falsenii*
			6	*no reliable identification*
1	4	*Microbacterium* spp.	2	*Microbacterium* spp.
			1	*Acidovorax* sp.
			1	*no reliable identification*
1	2	*Bergeyella* spp.	2	*no reliable identification*
4	15	*Cloacibacterium* spp.	15	*no reliable identification*
1	2	*Hydrogenophaga* spp.	2	*no reliable identification*

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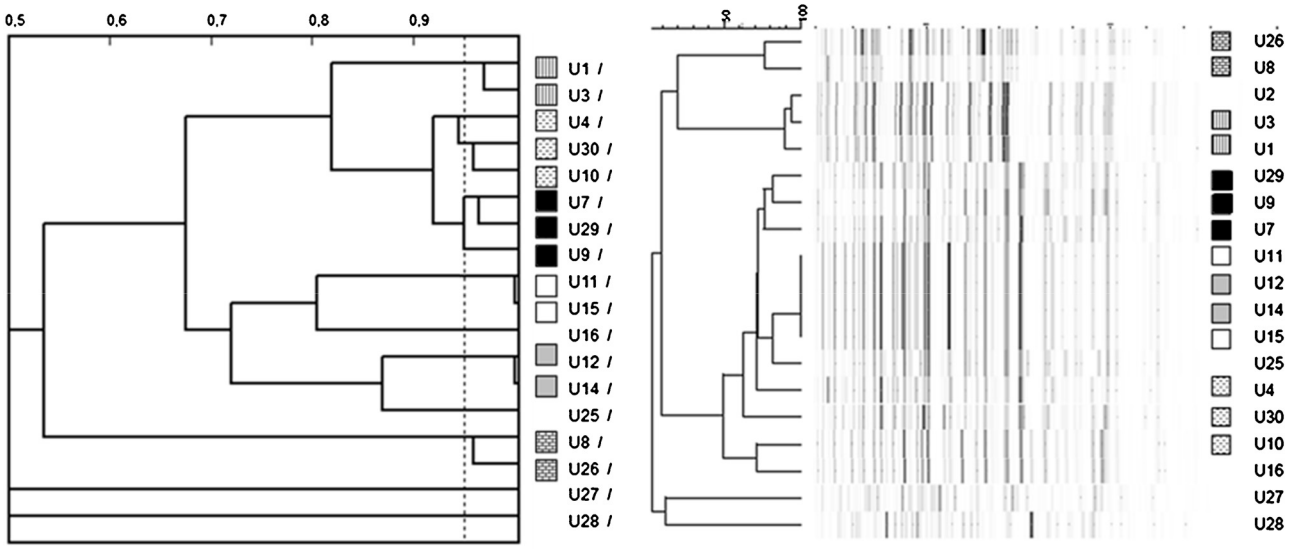

Fig. 1. Comparison between dendrograms derived from PhenePlate™ system profiles and MALDI–TOF spectra. On the left, we see a dendrogram derived from the phenotype profile from the PhenePlate™ system; whereas on the right, we see the dendrogram created from the mass spectra profile of the strains isolated at one point. The dendrograms were created using the Pearson correlation and UPGMA.

system. The predominant proteins of the mass spectra fingerprint correspond to ribosomal proteins due to their high abundance in bacterial cells (Croxatto et al., 2012). Therefore, the probable coevolution of the ribosomal proteins and rRNA may explain the similarities between the 16S rRNA genes (Ryzhov and Fenselau, 2001). MALDI–TOF and the PhP system method grouped the isolates consistently; similarly to the consistency between the PhP system and other molecular techniques such as Ribotyping, PFGE and RAPD (Ansaruzzaman et al., 2007, 1996; Kühn et al., 1995).

Of the commercial systems for the identification of bacteria, API is one of the most frequently used and implemented in many routine environmental laboratories. The API system has contributed to more effective management by enabling microbiologists to identify corresponding bacteria more rapidly; although compared to MALDI–TOF MS, the identification of large numbers of isolates through the use of API galleries is more tedious, more expensive and more time consuming. Nevertheless, phenotyping methods have inherent problems such as the corresponding databases being limited and the fact that not all the strains within a given species may exhibit a particular characteristic, which can depend on microbial growth and the substrate, thereby requiring 24 h for identification.

The obtained results support MALDI–TOF MS as a potentially useful and reliable alternative method for species identification in laboratories that receive many samples per day for bacteriological analysis. This is the case of water monitoring laboratories, which have to analyse samples from different points in drinking water treatment plants or distribution systems. Moreover, MALDI–TOF MS can produce results in just 10 min. As shown in this study, the major limiting factor in the use of MALDI–TOF MS for the identification of aquatic bacteria is the lack of a suitable mass spectra database, as most current databases focus on clinical species associated with human diseases. However, MALDI–TOF MS itself allows the creation a mass spectra fingerprint of the culturable fraction of the microbial populations of a given point within a treatment plant or distribution network, which could allow the detection of failures or population changes in the system quicker than other available techniques. The mass spectra fingerprints obtained can be compared and the isolates grouped accordingly. Consequently, its use could reduce the number of isolates to be further analysed which could then be incorporated into the database (Welker and Moore, 2011). In this way, gradually each company could develop its own library representing its own culturable bacterial community, which would represent just a minor part of the overall community. In addition, the incorporation of this methodology into routine analyses, which would enable generation of knowledge of the bacterial community, would constitute distinct added value for water companies. This methodology could provide companies with a traceability tool for water treatment processes and the corresponding distribution network, together with the possibility to detect failures in the system, the traceability of waterborne outbreaks and the characterization of intrinsic microbial populations.

Conclusions

MALDI–TOF MS is a promising method for the identification of bacteria in drinking water treatment plants. However, the major limiting factor is the lack of a suitable mass spectra databases; most current databases focus on clinically relevant species. For future environmental applications of MALDI–TOF MS, databases need to be expanded to provide more accurate results. MALDI–TOF MS is suited to both routine and frequent use, thanks to the speed, robustness and the simplicity of sample preparation as well as its method of result acquisition which incurs only minimal consumable costs, although it does require a considerable initial investment in the equipment. The discriminative capabilities of MALDI–TOF MS are equal or superior to those of other biochemical identification procedures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijheh.2016.01.001.

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4.4. Article 4

Heterotrophic monitoring at a drinking water treatment plant by matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometry after different drinking water treatments

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El coneixement de les comunitats bacterianes al llarg dels diferents tractaments en una estació de tractament d'aigua potable (ETAP) és indispensable per millorar el procés de potabilització i la gestió d'aquest tipus de plantes. Cada tractament que s'aplica a l'aigua exerceix una pressió selectiva sobre la comunitat microbiana, sent una barrera pels diferents microorganismes. Al llarg dels anys, els controls microbiològics s'han focalitzat en aquells microorganismes indicadors de la contaminació fecal. No obstant això, hi ha un desconeixement de les comunitats heteròtrofes presents en les diferents etapes de les plantes de tractament com també de la seva dinàmica al llarg de l'any. Les comunitats heteròtrofes de l'aigua, no indiquen la qualitat de l'aigua, tot i que, la monitorització d'aquestes comunitats permet la detecció d'errors en el sistema de tractament i també de recreixements de la població microbiana. Per tant, és necessari una tecnologia que permeti la monitorització en rutina d'aquestes comunitats i que pugui ser utilitzada en els propis laboratoris de les ETAPs.

L'objectiu d'aquest estudi va ser monitoritzar la comunitat heteròtrofa en les diverses etapes del procés de tractament mitjançant la tecnologia d'espectrometria de masses de desorció/ionització amb làser assistida per matriu acoblada amb un analitzador de temps de vol (MALDI-TOF MS), en dues estacions de l'any. L'estudi es va complementar amb el càlcul de la diversitat de Simpson i la similitud poblacional entre els comunitats aïllades en els diferents tractaments.

La caracterització de la comunitat heteròtrofa va tenir lloc a l'ETAP de Sant Joan Despí, que subministra aigua a una població de 1.200.000 habitants de l'àrea metropolitana de Barcelona. Per això, es va procedir a l'aïllament per filtració amb membrana de 0,45 μm, 30 soques de diferents mostres d'aigua, de l'aigua d'entrada a l'ETAP i de l'aigua de l'efluent després de diferents tractaments: filtració per sorra, ultrafiltració, osmosi inversa i finalment de l'aigua clorada. Les soques aïllades es van identificar amb la tecnologia MALDI-TOF MS (Bruker Daltonics GmbH). Posteriorment es van calcular els índexs de

diversitat i similitud poblacional mitjançant el sistema PhenePlate™ (PhPlate Microplate Techniques AB). El primer mostreig va tenir lloc durant l'hivern, mentre que el segon mostreig es va realitzar a l'estiu.

La comunitat heteròtrofa va experimentar una reducció de 4 unitats logarítmiques al llarg del procés de tractament. Un total de 133 soques es van aïllar a l'hivern i 144 soques a l'estació estival. Un total de 25 gèneres van ser identificats entre tots els punts analitzats, els fílums més abundants van ser, en ordre descendent: *Proteobacteria* (51%), *Firmicutes* (35%), *Bacteroidetes* (9%) i *Actinobacteria* (5%). Tot i el predomini de la classe *Gammaproteobacteria* i *Betaproteobacteria* en les primeres etapes del tractament, a mesura que el procés de tractament avançava, l'abundància relativa dels microorganismes *Proteobacteria* va disminuir. Contràriament, els microorganismes que pertanyien al fílum *Firmicutes* van augmentar. L'aigua d'entrada a la planta va ser la més diversa, i va ser lleugerament modificada després del llit filtrant de sorra i la ultrafiltració. La filtració per membrana d'osmosi inversa va produir un canvi en la comunitat. Finalment, la cloració va canviar dràsticament la comunitat que passà a ser dominada per soques del gènere *Bacillus*. A més, es van observar diferències estacionals entre les comunitats microbianes dels diferents punts estudiats. La diversitat de Simpson (Di) per les diferents comunitats analitzades va ser elevada, valors de Di > 0,82 en tots els casos. En relació a les identificacions de les soques mitjançant MALDI-TOF MS, els percentatges de soques identificades diferien segons el tractament i l'estació de l'any. Després d'alguns tractaments com la filtració per sorra i l'osmosi inversa, més de 50% de les soques aïllades no es van poder assignar a cap tàxon. Mentre que les soques aïllades a la ultrafiltració i a l'aigua tractada van obtenir els percentatges més alts d'identificació en els dos períodes analitzats.

En conseqüència, la tecnologia MALDI-TOF MS pot ser útil per monitoritzar la comunitat heteròtrofa aïllada en controls rutinaris de les estacions de tractament d'aigua. Així, s'obtindrà una visió de la comunitat pròpia de la planta. De fet, l'acumulació de dades de les comunitats heteròtrofes permetrà entendre la dinàmica de les poblacions i millorar la gestió de les plantes, com també la detecció de fallides en el sistema.

Heterotrophic monitoring at a drinking water treatment plant by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry after different drinking water treatments

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Short title: Heterotrophic monitoring by MALDI-TOF after different drinking water treatments

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Abstract

Monitoring natural resources, such as drinking water supplies, and treatment processes is essential to protect human health. The aim of this work was to assess the suitability of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) for routine heterotrophic monitoring of the different treatments performed in a drinking water treatment plant. Water samples were collected from raw surface water and after different treatments during two campaigns over a one-year period. Heterotrophic bacteria were studied and isolates were identified by MALDI-TOF MS. Moreover, the diversity index and the coefficient of population similarity were also calculated using biochemical fingerprinting of the populations studied. MALDI-TOF MS enabled us to characterize and detect changes in the bacterial community composition throughout the water treatment plant. Raw water showed a large and diverse population which was slightly modified after initial treatment steps (sand filtration and ultrafiltration). Reverse osmosis had a significant impact on the microbial diversity; while the final chlorination step produced a sharp shift in the composition of the bacterial community. Furthermore, the bacterial composition showed seasonal changes. Although MALDI-TOF MS could not identify all the isolates since the available MALDI-TOF MS database does not cover all the bacterial diversity in water, this technique could be used to monitor bacterial changes in drinking water treatment plants by creating a specific protein profile database for tracking purposes.

Keywords:

Drinking water; heterotrophic bacteria; identification; MALDI-TOF MS; water treatment

Introduction

In efforts to protect human health, drinking water is one of the most extensively monitored natural resources (Poitelon *et al*. 2009; Eichler *et al.* 2006). In drinking water treatment plants, water is subjected to different treatments so as to ensure adequate chemical and microbiological quality (Anonymous 1998). The different treatments exert a selective pressure which some groups of microorganisms can overcome and under certain conditions some can even grow. Fecal indicators and heterotrophic bacteria, among other microorganisms, should be monitored in the treated water supplied by drinking water treatment plants.

According to different drinking water regulations worldwide, fecal indicator bacteria (mostly *E. coli*) should be absent in 100 mL water to certify there is no fecal pollution. Furthermore, according to the Spanish regulations concerning drinking water (140/2003), the total aerobic and facultative anaerobic heterotrophic colony count (HPC) in treated water must not exceed 100 CFU/mL after incubation at 22 ºC for 3 days. Additionally, the HPC should be routinely monitored at 22 ºC so as to ensure that no abnormal changes in it occur during distribution. The HPC does not indicate the sanitary conditions of water, but it reflects the overall bacteriological quality of water resources and the drinking water treatment process. For example, when continuously monitored, changes in HPC may reflect failures in the water treatment or regrowth of microorganisms in water distribution systems (Diduch *et al.* 2016; Bartram *et al.* 2003; Allen *et al.* 2004).

Although not included in the regulations, some water treatment drinking companies are increasingly interested in gaining knowledge of the heterotrophic communities routinely present at each treatment step (personal communication). Knowledge of the heterotrophic microbial populations and their dynamics through the different treatments and under different temperature conditions could be used to rapidly detect failures and thereby further improve water management in drinking water treatment plants. Furthermore, such information could reflect on the activities of waterworks, because the populations could be associated with biofilm formation or bacterial regrowth. Although new genomic techniques allow us to determine the diversity of the dominant bacterial populations in water samples (Hong *et al.* 2010; Pinto *et al.* 2012), they do not provide the necessary information on the culturability of bacteria nor, consequently, on the bacteria which are routinely monitored by the HPC according to the current regulations.

The characterization of the HPC by traditional methods, such as biochemical test galleries, is extremely time consuming and therefore a novel methodology for routine water monitoring is needed. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) has emerged in recent years as a rapid proteomic method for the identification of bacteria from clinical settings (Welker 2011). The cost of the equipment is high, although there

is minimal consumable cost after the initial investment (Bizzini & Greub 2010). MALDI-TOF MS is a library-dependent method based on spectral analysis of bacterial proteins, mainly ribosomal, using intact cells with no previous treatment (Welker 2011). It has been reported that the current commercial MALDI-TOF database could identify some bacterial strains from water samples (Sala-Comorera *et al.* 2016). The aim of this work was to assess the suitability of MALDI-TOF MS for routine heterotrophic bacteria monitoring of the different treatments performed in a drinking water treatment plant. The study was performed considering two different seasons, and it was complemented with the assessment of the diversity and population similarities using a biochemical phenotypic method (PhenePlate^{TM} system).

Methods

Sampling

Samples were collected from a drinking water treatment plant in Spain (Sant Joan Despí - Barcelona). In the plant (Fig. 1), surface water from the River Llobregat is pre-oxidized using chlorine dioxide and suspended solids are removed by settling, followed by sand filtration. Then, groundwater is incorporated into the treatment process. From here, the water may be treated using two processes: one includes ozonization and granular activated carbon filtration to oxidize and remove organic constituents and residual disinfectants; the other includes the use of ultrafiltration and reverse osmosis. The two lines converge in a mixing chamber. Eventually, the water is chlorinated before leaving the drinking water treatment plant. The resultant disinfected water enters a distribution system which supplies 21 municipal pipeline distribution networks. The treatment plant serves a population of 1,200,000 from the Barcelona metropolitan region.

Samples were collected from 5 different stages of the water treatment process: raw surface water (R), after sand filtration (S), after membrane ultrafiltration (U), after reverse osmosis (O) and from the drinking water reservoir after the chlorination (T). The first sampling was performed in winter, when the temperature of the water was at its lowest (around 13 ºC); while the second was carried out in summer, when the temperature of the water was at its highest (around 22 ºC).

Volumes of water between 0.001-0.1 mL from raw surface and sand filtration water, 100-350 mL from ultrafiltration and reverse osmosis water and 1 L of treated water were filtered so as to obtain isolates. Immediately after sampling, sterile sodium thiosulfate was added so as to

neutralize chlorine from treated water. Water samples were kept cold before filtration and they were analysed within 3 h of collection. Membrane filtration was performed using 0.45 µm pore size membranes (Millipore, Germany), following standard techniques. The membranes with the filtrated samples were incubated on Water Plate Count Agar (ISO) plates (Oxoid, United Kingdom) at 22 ºC ± 2 ºC for 3 days. The medium was appropriate for the cultivation of microorganisms from water, according to ISO 6222:1999 - Water quality: Enumeration of culturable micro-organisms, colony count by inoculation in a nutrient agar culture medium. After incubation, 30 strains from each step were isolated from the plates to assess the heterotrophic community diversity as previously reported (M. A. G. Bianchi and Bianchi, 1982). The isolates were sub-cultured on the same medium.

Mass spectrometry analysis

All the isolated strains were analysed by MALDI-TOF MS using the procedure detailed below. A small quantity of the colony growth on the Water Plate Count Agar (ISO) was transferred to a metallic MALDI-TOF MS plate and 1 µL of matrix (saturated alpha-cian-4-hydroxycinamic acid-50% acetonitrile-2.5% trifluoroacetic acid) was added to cover the specimen preparation. The plate was then left to dry for 5 min at room temperature. Measurements were performed using a Mircroflex II mass spectrometer (Bruker Daltonics GmbH, Germany) equipped with a 60 Hz laser. The proteic spectra obtained with the spectrometer were processed using the Bruker MALDI Biotyper v2.0 software (Bruker Daltonics GmbH, Germany) and compared with the spectrum database. The identification score criteria used were those recommended by the manufacturer: < 1.700 was interpreted as not a reliable identification; 1.700 to 1.999 indicated identification at the genus level; while a score ≥ 2.000 identified the isolates at the species level.

Indices of population diversity and similarity

Simpson's diversity index (Di) was used to calculate the diversity of the bacterial populations at the different sampling points and for each season (Hunter & Gaston 1988). Di is a relative measure of the distribution of isolates into different phenotypes; while the similarity between populations was calculated using the coefficient of population similarity (Sp) (Kühn *et al.* 1991). The populations are considered similar when the Sp value is greater than 0.2 (Kühn *et al.* 1991). The diversity indexes were calculated using the biochemical profiles obtained with the miniaturized biochemical phenotyping method, the PhenePlate™ system, PhP-48 plates (PhPlate Microplate Techniques AB, Sweden) as previously described (Casanovas-Massana & Blanch 2012). Briefly, PhenePlate™ system is a miniaturized biochemical phenotyping method based on evaluation of the kinetics of several biochemical reactions performed in microtiter

plates. The comparison of the bacterial populations from the various samples was analysed using the unweighted-pair group method analysis (UPGMA) with average linkage. The index calculations were performed using the software PhPWin® (PhPlate Microplate Techniques AB, Sweden).

Results

The water treatment plant showed a reduction of around 4 $\log_{10}$ units in the HPC (Table 1). Sand filtration reduced the HPC in 1-2 $\log_{10}$ whereas no significant changes were observed after ultrafiltration treatment. After reverse osmosis, the counts dropped sharply and, although reverse osmosis filtration was expected to eliminate all bacterial cells, heterotrophic bacteria were still detected. Raw and treated water showed similar values in both seasons, whereas the concentration after sand filtration, ultrafiltration and reverse osmosis was between 0.5-1 $\log_{10}$ units higher in summer than in winter. A total of 277 colonies were isolated from the water treatment process: 133 isolates corresponding to winter and 144 to summer.

Identification of the isolates by MALDI-TOF MS

All the isolates representing the microbial cultivable communities suspended in the water column after the different treatments applied in the water treatment plant were characterized by MALDI-TOF MS. This methodology allowed the identification of the isolates into different levels of classification from phylum to species. The dominant phyla (Fig. 2) for all the sampling sites combined were, in descending order: *Proteobacteria* (51%), *Firmicutes* (35%), *Bacteroidetes* (9%) and *Actinobacteria* (5%). Despite the dominance of *Proteobacteria* in the first stages, when moving along the water treatment process, the relative abundance of *Proteobacteria* decreased. In contrast, the relative abundance of *Firmicutes* increased sharply in both seasons, becoming the dominant population after chlorination.

In winter sampling (Fig. 2), at least 85% of the isolates retrieved from the raw water and the first steps (sand filtration and ultrafiltration) were assigned to *Betaproteobacteria* (20%) and *Gammaproteobacteria* (65%). The number of isolates belonging to the *Proteobacteria* phylum decreased after the ultrafiltration treatment. Among the strains isolated after the ultrafiltration, an increased abundance of isolates affiliated to the phylum *Bacteroidetes* was observed, yet no

member of this phylum was observed in the raw water or after sand filtration. Members of the phylum *Actinobacteria* were only isolated in the ultrafiltration step. *Firmicutes,* which represented less than 10% of the community in the early treatments, represented 96% of the community after the chlorination step.

In summer sampling (Fig. 2), the isolates affiliated to the *Proteobacteria* phylum represented a smaller part of the overall community (63%) than in winter. *Betaproteobacteria* represented 20% of the isolates, *Gammaproteobacteria* 41% and *Alphaproteobacteria* 2%, this class was not identified in winter sampling. *Betaproteobacteria* greatly increased in the reverse osmosis, and constituted 69% of the isolates after this step. During sand filtration and ultrafiltration, the content of *Bacteroidetes* was relatively stable; this phylum was not detected in the other stages of the treatment. *Actinobacteria* abundance increased in the successive treatments from raw water to the reverse osmosis, accounting for nearly 20% of the reverse osmosis effluent community; however, it was not present after the ultrafiltration step. The isolates affiliated to the *Firmicutes* phylum dominated the community of the treated water, as in winter sampling.

MALDI-TOF MS analysis allowed us to identify at genus or species level 65% of all the strains isolated from the water samples according to the database available. The percentage of the genera identified at each treatment and the percentage of not identified are shown in Table 2. The percentages of the identifications differed from treatment and seasons. After some treatments, sand filtration and reverse osmosis, more than 50% of the isolates could not be identified to any taxon. Whereas the isolates from ultrafiltration and treated water in both seasons obtained the highest score and isolates could be classified with high confidence. Regarding the identification level (species or genus), a larger number of strains from raw water, sand filtration and ultrafiltration were classified at species level in both seasons. Whereas the majority of isolates from reverse osmosis and treated water could only be classified at genus level.

A total of 25 genera were identified from all the sampling locations and, in general, a decrease in genus diversity was observed along the water treatment procedures (Table 3). However, after ultrafiltration, an increase in genus diversity was found in both samplings in comparison with the number of genera identified in the previous step. It should be noted that the percentage of not identified isolates after sand filtration was high, for this reason the number of genera isolated did not represent the real diversity. Raw water showed the highest population diversity (12 genera). Most of the genera were detected in both seasons, such as *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Bacillus* and *Ralstonia.* Genera such as *Janthinobacterium*, *Pantoea* and *Shewanella* were isolated in winter; whereas *Dickeya, Lysinibacillus, Massilia*

and *Micrococcus* were identified in summer. Some of the isolates identified after sand filtration were also present in the raw water, but 2 different new genera were detected in sand filtrated water in winter: *Pseudomonas* sp. and *Staphylococcus* sp. Meanwhile, in the corresponding summer isolates, 3 new genera were present: *Chryseobacterium, Elizabethkingia* and *Staphylococcus.* Following water treatment, the majority of the genera identified in the ultrafiltration effluent were also observed in the sand filtration effluent and the raw water. Members of *Pseudomonas* sp. were numerically the most frequently encountered in the winter community; whereas the community in the summer was composed of members of different genera, primarily *Acidovorax* sp., *Aeromonas* sp., *Elizabethkingia* sp. and *Pseudomonas* sp. The number of genera identified in the reverse osmosis effluent was greatly reduced compared to the samples from the early treatment stages. Reverse osmosis reduced the microbial diversity to 4 genera in each season; the community was dominated by *Flavobacterium* sp. and *Pseudomonas* sp. in the winter and *Acidovorax* sp. in the summer. Chlorination resulted in a selective pressure that permitted only a few genera of bacteria to overcome the treatment; two per season. *Bacillus* sp. was the largest group found in the treated water in winter, together with *Kocuria* sp.; whereas *Bacillus* sp. and *Lysinibacillus* sp. were the most common in summer.

The MALDI-TOF MS analysis also allowed us to identify some isolates at the species level. A total of 40 species belonging to 25 genera were identified, most of them isolated once or twice at the different sampling sites (Table 4). The heterotrophic community of the treatment plant was composed of a large number of species, each of which represented a small fraction of the total community. The heterotrophic community underwent changes not only at the phylum level, but also with respect to the predominant genera and species. It should be noted that some species were found after at least three different consecutive treatments, but these species were different in each season: we identified *Janthinobacterium lividum* among the winter isolates, *Acidovorax temperans* in summer.

Indices of population diversity and similarity

The isolates were further typed biochemically for comparison using the PhenePlate™ system, so as to calculate the Simpson diversity index, Di, of the samples. However, only 174 out of 277 isolates could be subjected to clustering and population analysis, because a total of 103 isolates were regarded as non-typeable, due to their weak or very weak reaction with the PhenePlate™ system plates. All the isolates belonged to the *Bacillus* genus, which were isolated from the treated water were non-typeable by PhenePlate™ system.

A high value of Di (maximum 1) indicates an even distribution of the isolates into many different biochemical phenotypes; whereas a low value (minimum 0) indicates there are few biochemical phenotypes that dominate the population. High values of Di (> 0.82 in all cases) were found for the sampling sites in both seasons (Table 5). Consequently, there was no dominant phenotype among the populations. The diversity of the bacterial community in the raw water was almost identical in both seasons and it was particularly rich. Di decreased slightly over the successive treatments the water was subjected to during winter, whereas in the summer, the diversity decreased after sand filtration and then it remained stable over the following treatments.

The heterotrophic community associated with the winter sampling were significantly different at the stages analysed, according to the Sp values, which were lower than 0.2 (Fig. 3). The difference in clustering could be ascribed to the variation in relative abundance of the genera among treatments. For the summer samples, the highest similarity indexes were recorded after sand filtration, ultrafiltration and reverse osmosis, with Sp values above 0.2 (Fig. 3). Although variations in the percentages of genera were observed in the summer, there were no significant changes in either the composition or structure of these populations as shown by the clustering analysis and similarity population indexes. Low similarity was observed in the raw samples between seasons (Sp =0.08). Consequently, the heterotrophic community of the water that was treated in the plant was not constant during the year.

Discussion

The present study explores the utilization of a novel technology MALDI-TOF MS so as to characterize the heterotrophic bacteria isolated in routine analyses after five treatment steps in a drinking water treatment plant in two moments of the year (when the temperature of the water was at its lowest and highest). The characterization showed a shift from predominantly Gram-negative bacteria in the raw water and the drinking water treatment steps to mostly Gram-positive bacteria in the chlorinated water in both samplings: winter and summer. The obtained results showed that the phylum *Proteobacteria* dominated within the different communities. This observation is in agreement with previous studies, which have demonstrated the preponderance of members of the *Proteobacteria* phylum in drinking water systems (Poitelon *et al.* 2009; Pinto *et al.* 2012; Poitelon *et al.* 2010). Therefore, members of the *Betaproteobacteria*

class are particularly sensitive to chlorination (Poitelon *et al*. 2010), which could explain the decrease in *Betaproteobacteria* with the progress of the water treatment.

The characteristics of treated water depend on the water source, the treatment processes, storage and distribution devices (Vaz-Moreira *et al.* 2013; Eichler *et al.* 2006). No water processing step affected each group studied identically. The obtained results by MALDI-TOF MS complemented with the performed biochemical fingerprinting support changes on the heterotrophic composition and structure of heterotrophic communities throughout the water treatment. This supports that this methodology is valid to detect changes among the heterotrophic community in the different treatment steps. Previous studies have shown that after flocculation and sand filtration no major changes in the structures occurred (Eichler *et al.* 2006; Tian *et al.* 2014). However, other studies have stated that established microbial communities on the surfaces of biofiltration materials, such as sand or granular activated carbon, affect the microbial community in the effluent water (Lautenschlager *et al.* 2014); which is in agreement with the obtained results showing that communities after sand filtration differed from those in the raw water. A chemical disinfection step is useful in water treatment plants so as to avoid the regrowth of microorganisms, especially pathogens. In this study, chlorination greatly changed the structure of the bacterial community and plays a substantial role in determining its final composition. Even though disinfection is effective against the vast majority of the indigenous bacteria, the *Firmicutes* phylum remains functional. This is in agreement with previous studies in which *Firmicutes* also dominated after disinfection treatment (Norton & LeChevallier 2000; Poitelon *et al.* 2010).

Concerning the identification of the isolates, some of them belonged to genera that had already been reported in drinking water treatment plants or drinking water distribution systems. Although previous studies used other culture or culture-independent methods so as to isolate or characterize the community, genera identified by MALDI-TOF MS in the present study are in agreement with these studies. To the best of our knowledge this is the first study in which the heterotrophic community is reported by MALDI-TOF MS. Accordingly, previous studies have reported the isolation of members of the genera *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Comamonas*, *Chryseobacterium*, *Flavobacterium*, *Janthinobacterium*, *Micrococcus*, *Pseudomonas* and *Ralstonia* from river or fresh water from Netherlands or USA (Bereschenko *et al.* 2008; Vaz-Moreira *et al.* 2011; Norton & LeChevallier 2000). Meanwhile, genera such as *Acidovorax, Camamonas, Flavobacterium* and *Pseudomonas* have previously been identified in granular activated carbon effluent *(*Norton & LeChevallier, 2000*;* Poitelon *et al.* 2010) and *Acidovorax, Flavobacterium, Janthinobacterium, Pseudomonas* after ultrafiltration (Bereschenko *et al.* 2008). Members affiliated to *Acidovorax, Janthinobacterium* and

Pseudomonas have also been identified as part of the biofilm attached to membranes of reverse osmosis (Bereschenko *et al.* 2008).

Biochemically clustering by PhenePlate^{TM} system gave additional information so as to understand the similarities between microbial communities in both snapshots from different seasons. High levels of diversity were found in all the samples, which was in agreement with MALDI-TOF MS results, confirming the complexity of the microbial community. Some bacterial species were removed during the treatment process, so bacterial species and the diversity decreased as expected throughout the treatments. Whereas, some bacterial species stated above were found consecutively in different treatments. In future, after more heterotrophic routine monitoring, these isolates could be used as a tracking tool for the efficiency of some treatments. In contrast to the rest of the samples, the high Sp value obtained for populations at 3 sampling points in the summer revealed that a similar community is established after the sand filtration, ultrafiltration and reverse osmosis treatments in the summer. This could be explained by biofilms forming in the network that distributes the water to the different treatments, which could influence the microbial populations in the water column.

The applied MALDI-TOF MS allowed a rapid characterization of the heterotrophic communities after each treatment step, and it would therefore be an appropriate method for the rapid monitoring of the cultivable community in a drinking water treatment plant in future. However, in this characterization of the heterotrophic community, an important percentage of the isolates could not be identified, so the creation of each own database of microorganisms would be necessary because the current protein profile database do not cover the bacterial diversity found in water samples. For example, a high number of isolates could not be identified especially in some treatments, such as sand filtration and reverse osmosis. MALDI-TOF MS system has a further advantage that the mass spectra from unidentified isolates could be added to the database. This methodology could be also useful to identify recurrent or abnormal strains isolated in routine could be identified in minutes, with the regular resources of a laboratory of any waterworks company. Better knowledge of the microbial community will provide the opportunity for improved management of drinking water plants, since all the decisions at the different stages could determine the final bacterial composition.

Conclusions

MALDI-TOF MS is a useful technique so as to rapidly characterize the heterotrophic microorganisms which are routinely detected by routine culture techniques in a drinking water treatment plant. Monitoring and characterizing these communities with MALDI-TOF allowed mapping of the own community, which is different depending on the water temperature during the year. Accumulating data on heterotrophic communities is important to further understand the dynamics and ecology of the populations in the different processes in a drinking water treatment plant. Furthermore, identification of the own heterotrophic communities may help detection of failures in the treatment systems.

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Conflict of interest

No conflict of interest declared.

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Tables and figures

Table 1. Seasonal HPC at the different treatment steps and the distribution of the isolates used in the study. Values of standard deviation for colony counts are indicated in brackets.

Step	Winter		Summer	
	$\log_{10}$ (CFU mL^{-1})	**No. of isolates**	**$\log_{10}$ (CFU mL^{-1})**	**No. of isolates**
Raw water (R)	4.66 (±0.55)	26	4.50 (±0.51)	30
After sand filtration (S)	2.27 (±0.23)	24	3.05 (±0.67)	25
After ultrafiltration (U)	2.34 (±0.34)	25	2.95 (±0.41)	29
After reverse osmosis (O)	0.97 (±0.32)	29	1.32 (±0.25)	30
After chlorination treatment (T)	< 0.03	29	< 0.13	30

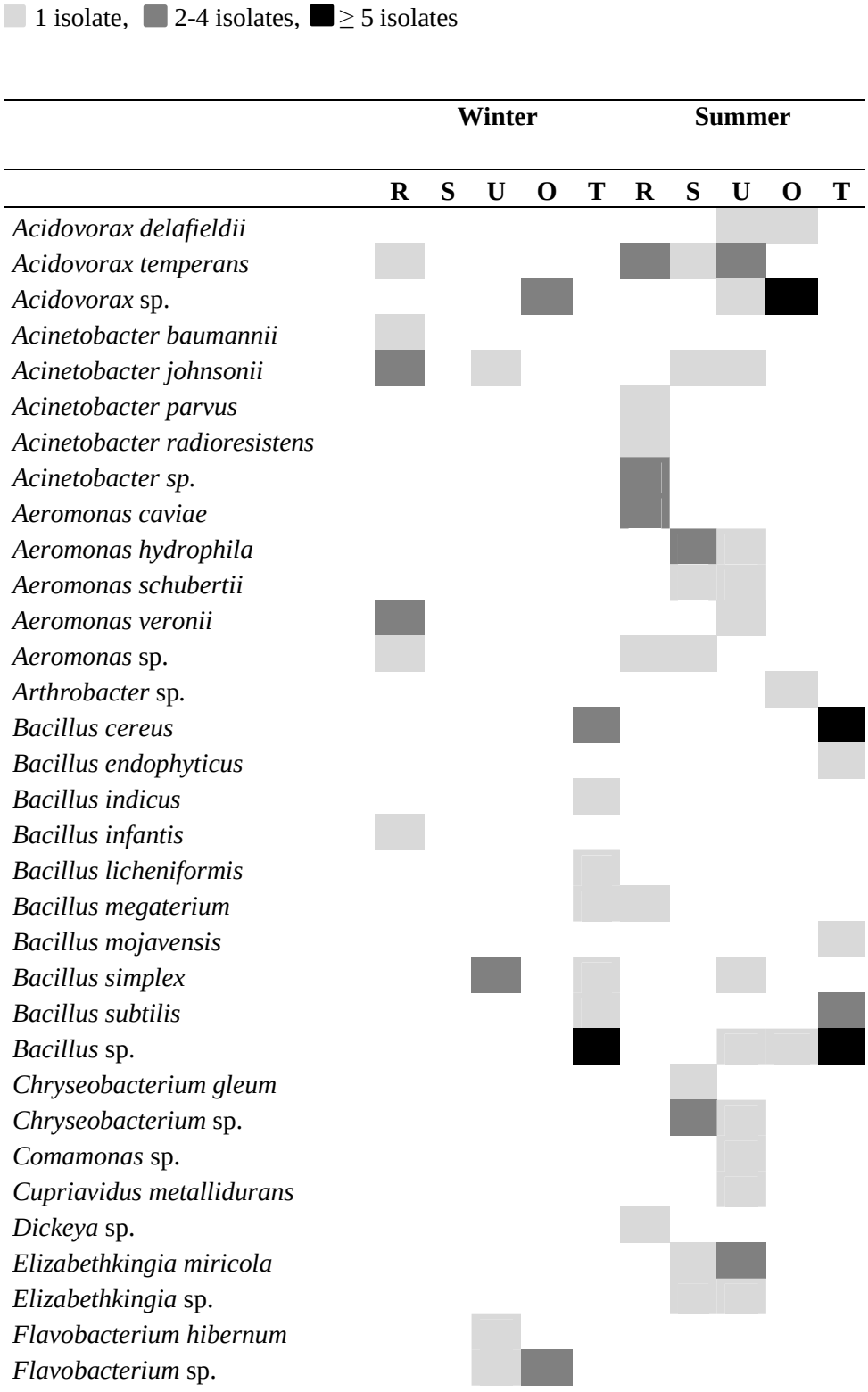
Table 2. Percentages of isolates identified at taxonomic levels by MALDI-TOF MS at each treatment step. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water.

	Winter					**Summer**				
	R	**S**	**U**	**O**	**T**	**R**	**S**	**U**	**O**	**T**
Species	58	21	72	17	34	37	44	52	3	50
Genus	8	17	20	21	59	20	20	21	40	37
No reliable identification	34	62	8	62	7	43	36	27	57	13

Table 3. Seasonal distribution of the heterotrophic community after the different treatment steps based on MALDI-TOF MS spectra at the genus level. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water.

	% Community in:									
	Winter					**Summer**				
	R	**S**	**U**	**O**	**T**	**R**	**S**	**U**	**O**	**T**
Acidovorax sp.	4			7		8	4	14	30	
Acinetobacter sp.	15		4			13	4	3		
Aeromonas sp.	15					18	16	11		
Arthrobacter sp.									3	
Bacillus sp.	4		8		90	3		8	3	70
Chryseobacterium sp.							16	3		
Comamonas sp.								3		
Cupriavidus sp.								3		
Dickeya sp.						3				
Elizabethkingia sp.							8	11		
Flavobacterium sp.			8	14						
Janthinobacterium sp.	4	13	12	3						
Kocuria sp.					3					
Lysinibacillus sp.						3				17
Massilia sp.						3				
Microbacterium sp.									7	
Micrococcus sp.			4			3	12			
Pantoea sp.	4									
Pseudomonas sp.		13	52	14				11		
Ralstonia sp.	8					3				
Serratia sp.			4							
Shewanella sp.	12	8								
Sphingopyxis sp.								3		
Staphylococcus sp.		4					4			
Wautersiella sp.								3		
no reliable identification	34	62	8	62	7	43	36	27	57	13

Table 4. Number of isolates identified by MALDI-TOF MS at each point, at the species (cut-off score ≥ 2.000) and genus (cut-off score ≤ 2.000) levels. The genus of the isolates that could not be identified at the species level is indicated with sp. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water.



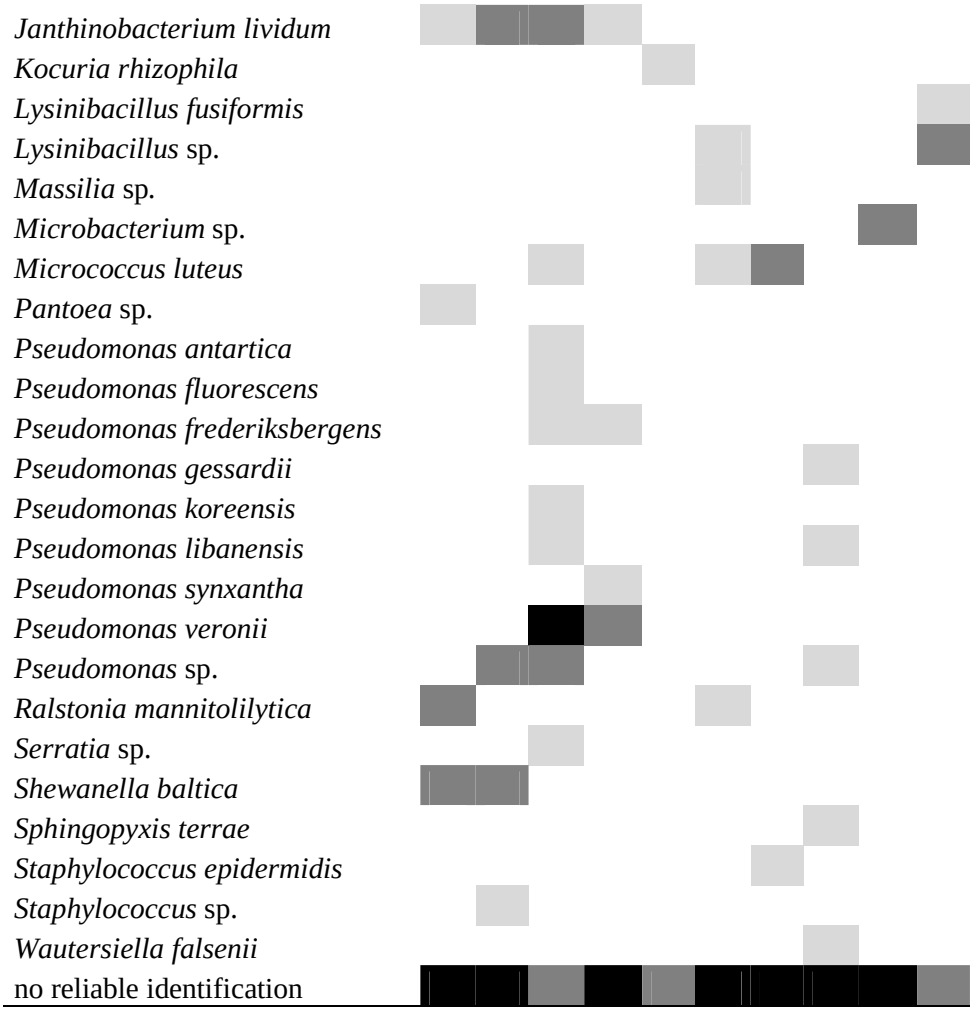

Janthinobacterium lividum
Kocuria rhizophila
Lysinibacillus fusiformis
Lysinibacillus sp.
Massilia sp.
Microbacterium sp.
Micrococcus luteus
Pantoea sp.
Pseudomonas antartica
Pseudomonas fluorescens
Pseudomonas frederiksbergens
Pseudomonas gessardii
Pseudomonas koreensis
Pseudomonas libanensis
Pseudomonas synxantha
Pseudomonas veronii
Pseudomonas sp.
Ralstonia mannitolilytica
Serratia sp.
Shewanella baltica
Sphingopyxis terrae
Staphylococcus epidermidis
Staphylococcus sp.
Wautersiella falsenii
no reliable identification

Table 5. Simpson Diversity of the heterotrophic populations analysed for both seasons. ND: not determined. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water.

Population	Winter Diversity (Di)	Summer Diversity (Di)
R	0.98	0.97
S	0.97	0.87
U	0.94	0.88
O	0.82	0.88
T	ND	ND

Figure 1. Scheme of the water drinking treatment plant

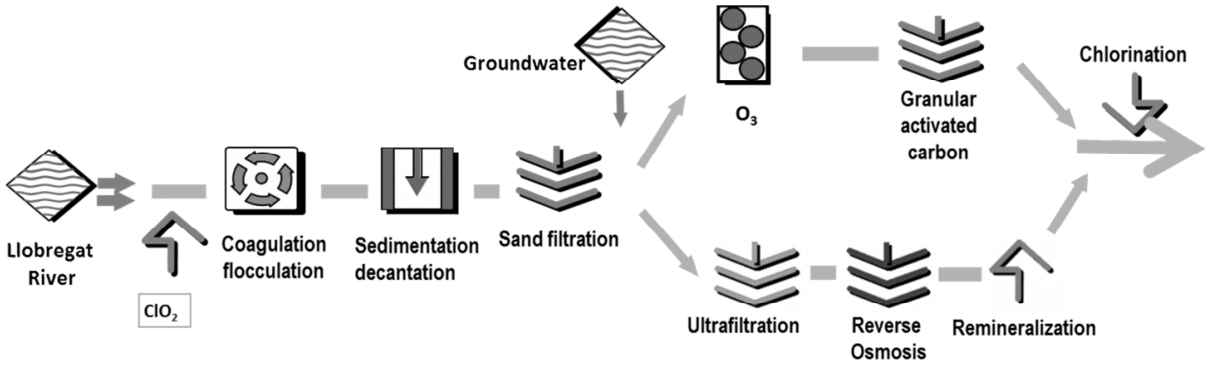


Figure 2. Relative abundance of bacterial phyla at the five sampling locations in each season. The dominant phylum, *Proteobacteria*, is divided into classes: *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water.

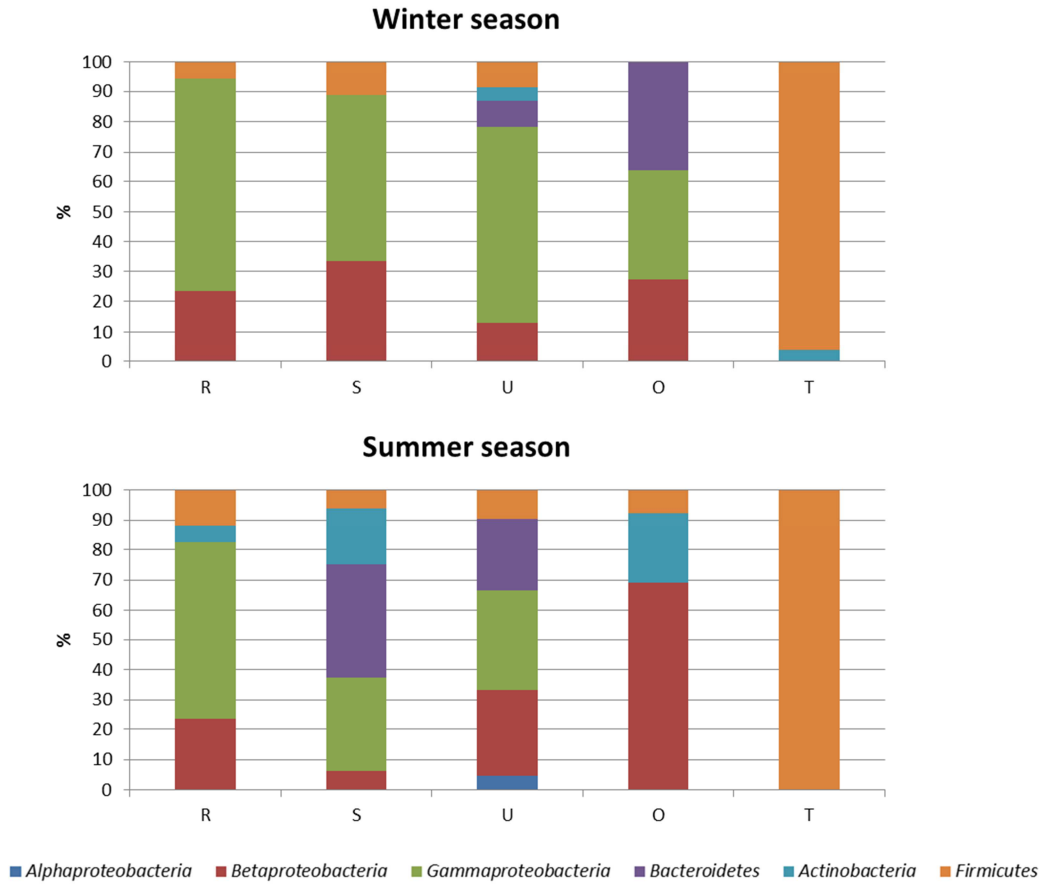
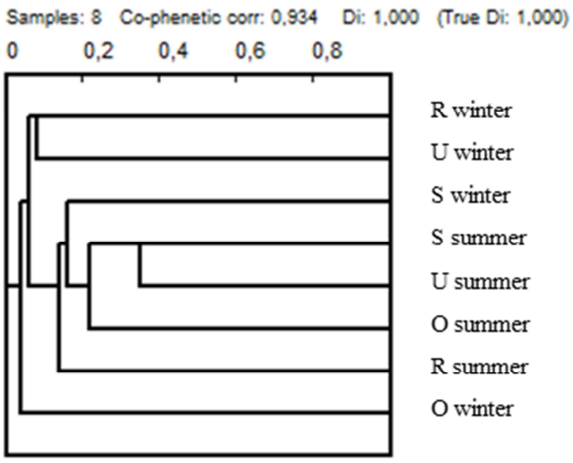


Figure 3. Dendrogram of the clustering analysis of the similarity of populations (Sp) for each treatment step. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water.



4.5. Article 5

Pseudomonas-related populations associated with reverse osmosis in drinking water treatment

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La tecnologia de filtració per membrana d'osmosi inversa (OI) s'ha implantat en el camp del tractament d'aigua, tant en plantes dessalinitzadores com en estacions de tractament d'aigües potables (ETAPs), per tal de proporcionar aigua de major qualitat. Tanmateix, la formació de biofilms en la superfície de les membranes, coneguda com *biofouling*, contribueix al deteriorament de les membranes, fet que genera efectes adversos des d'un punt de vista operatiu i econòmic. El biofilm de les membranes OI pot albergar diferents patògens, entre ells *Pseudomonas aeruginosa.* Tot i que la filtració per OI permet retenir els microorganismes, es segueix observant microorganismes en el medi GSP (glutamat midó de fenol) agar a l'aigua osmotitzada. Aquest medi és selectiu per *Pseudomonas* i *Aeromonas,* i és emprat per a detectar la presència de *P. aeruginosa*, ja que a l'aigua destinada al consum humà no ha de contenir aquest microorganisme, segons la normativa vigent. La capacitat metabòlica adaptativa del gènere *Pseudomonas* explica la seva ubiqüitat en diferents ambients, a més, aquest gènere ha estat descrit com un bon indicador de recreixement bacterià en sistemes d'aigua potable de l'àrea de Barcelona.

L'objectiu d'aquest estudi va ser caracteritzar la comunitat bacteriana planctònica de l'aigua d'alimentació de la OI i de l'aigua osmotitzada aïllada en el medi GSP agar. També es va avaluar la capacitat de les soques aïllades de formar biofilm *in vitro* i la seva susceptibilitat antimicrobiana.

Amb aquest objectiu es van realitzar 4 mostreigs en dos anys consecutius: 2 quan la temperatura de l'aigua era elevada, 22 ˚C i 2 quan la temperatura de l'aigua era baixa, 12 ˚C. A cada mostreig es va procedir a l'aïllament de 25 soques de l'aigua d'alimentació de la OI i 25 soques de l'aigua osmotitzada. Les soques aïllades van ser identificades mitjançat MALDI-TOF MS (Bruker Daltonics GmbH) i es van fenotipar bioquímicament amb el sistema PhenePlate™ (PhPlate Microplate Techniques AB) per tal de calcular els índexs de similitud poblacional entre les comunitats de l'aigua d'abans i després de l'osmosi. Posteriorment, es va avaluar la capacitat de formació de biofilm d'aquelles soques

representants dels grups fenotípics mitjançant l'assaig en microplaca amb tinció de cristall violeta. Paral·lelament, les soques representats també es van sotmetre a un assaig de susceptibilitat antimicrobiana de 10 antibiòtics mitjançant un test de difusió en disc.

Un total de 191 soques van ser aïllades. En aquest estudi es va observar que les comunitats planctòniques de l'aigua d'alimentació i de l'aigua osmotitzada eren diferents (Sp<0,2) segons el coeficient de similitud poblacional, malgrat que compartien algun grup fenotípic. En canvi, les comunitats del mateix punt d'aïllament i temperatura eren similars en els dos mostreigs. Les comunitats de l'aigua d'alimentació a temperatura alta eren més diverses que les comunitats de l'aigua osmotitzada i de l'aigua d'alimentació a temperatura baixa, dominades principalment pels gèneres *Pseudomonas* i *Acidovorax.* L'assaig espectrofotomètric de la formació de biofilm que es realitzà amb les soques representants dels 19 grups fenotípics va revelar que només quatre soques tenien la capacitat de formar biofilm *in vitro*. Les soques amb capacitat de formar biofilm es van aïllar tant en l'aigua d'abans de l'OI com després, per tant aquestes soques poden estar relacionades en el deteriorament de les membranes. Un 95% dels aïllats va presentar resistència (incloent resistència intermèdia) als antibiòtics: aztreonam, cloramfenicol i cefotaxima. Les resistències més freqüents de les soques del gènere *Pseudomonas* van ser als antibiòtics aztreonam, cloramfenicol, cefotaxima i trimetoprima+sulfametoxazol. *Stenotrophomonas maltophilia* va mostrar resistència a la majoria dels antibiòtics testats. La soca d'*Acidovorax delafieldii* va ser susceptible a tots els antibiòtics assajats, mentre que les soques que pertanyien als gèneres *Ensifer* i *Acinetobacter* van mostrar un perfil de resistència als antibiòtics gairebé idèntic a 4 antibiòtics. El patró de resistència a antibiòtics va estar més lligat a les espècies que no en el punt d'aïllament de les soques.

En conclusió, existeixen diferències entre les comunitats de l'aigua abans i després de la membrana d'OI, indicant una correcta filtració a nivell d'OI i una posterior recolonització de l'aigua osmotitzada. En cap cas, es va detectar el patogen oportunista *P. aeruginosa*, complint així els paràmetres de qualitat microbiològica de l'aigua de consum. D'altra banda, la presència de soques amb capacitat per formar biofilm com també la presència de soques resistents a antibiòtics poden contribuir a la formació de *biofouling* com també a la dispersió de la resistència a antibiòtics.

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Research article

Pseudomonas-related populations associated with reverse osmosis in drinking water treatment

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ABSTRACT

Reverse osmosis membrane filtration technology (RO) is used to treat drinking water. After RO treatment, bacterial growth is still observed in water. However, it is not clear whether those microorganisms belong to species that can pose a health risk, such as *Pseudomonas* spp. The goal of this study is to characterize the bacterial isolates from a medium that is selective for *Pseudomonas* and *Aeromonas* which were present in the water fraction before and after the RO. To this end, isolates were recovered over two years and were identified by matrix-assisted laser desorption/ionization–time of flight mass spectrometry. They were then biochemically phenotyped and the population similarity indexes were calculated. The isolates were analysed for their capacity to form biofilms *in vitro* and antimicrobial susceptibility. There were significant differences between the microbial populations in water before and after RO. Furthermore, the structures of the populations analysed at the same sampling point were similar in different sampling campaigns. Some of the isolates had the capacity to form a biofilm and showed resistance to different antibiotics. A successful level filtration via RO and subsequent recolonization of the membrane with different species from those in the feed water was found. *Pseudomonas aeruginosa* was not recovered from among the isolates. This study increases the knowledge on the microorganisms present in water after RO treatment, with focus in one of the genus causing problems in RO systems associated with human health risk, *Pseudomonas*.

1. Introduction

The aim of implementing reverse osmosis membrane technology (RO) in a water treatment plant is to obtain high-quality water. RO efficiently removes of a wide variety of water contaminants: microbial constituents, and both organic and inorganic compounds. RO has been widely used to date. However, the major limitation of this technology is the fouling of the membranes, which limits its application. This fouling is a complex process involving biofilm formation, along with the deposition of solute or particles in the feed water (Herzberg and Elimelech, 2007; Kang and Cao, 2012). Besides biofilms contributing to membrane deterioration, biofilms growing in water distribution systems are also associated with human health risks, since they can harbour and be reservoirs of opportunistic pathogens. Some such pathogens isolated from biofilms in potable water distribution systems include *Mycobacterium avium, Pseudomonas aeruginosa, Klebsiella* spp., *Legionella* spp. and *Flavobacterium* spp. (Norton and LeChevallier, 2000; Ridgway and Olson, 1981; Walker et al., 1993).

During drinking water analysis in treatment plants, bacterial growth has often been observed in permeate water after RO using GSP (glutamate starch phenol) agar (Kielwein, 1969): a selective medium for *Pseudomonas* and *Aeromonas*. Considering that this water is intended for human consumption via a distribution network, it should be free of microbial indicators (*Escherichia coli* and *Enterococcus*) and certain water quality regulations even require the absence of *P. aeruginosa*. According to the European Council Directive 98/83/EC, water for consumption in bottles or containers must be free of *P. aeruginosa*, since its presence constitutes a health risk. *P. aeruginosa* is an opportunistic pathogen associated with waterborne diseases (Römling et al., 1994; Warburton, 1993). The species can grow in environments with low nutrient content (Moreira et al., 1994) and it is an indicator of

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other opportunistic pathogens (De Victorica and Galván, 2001; Geldreich, 1992). *Pseudomonas* spp. have been detected in most biofilms on membranes in RO systems (Bereschenko et al., 2008; Kwon et al., 2011) and distribution networks (Ren et al., 2015).

Few studies have focused on the microbiology of water after RO treatment. In a recent study, Meneses and Flores (Meneses and Flores, 2016) showed a reduction in the bacterial load by 1.5 $\log_{10}$ CFU ml^{-1} after RO treatment, achieving the microbiological standards for water quality. In another study, more than 99% of bacterial cells were retained by the membrane, obtaining counts of <50 cells/ml (Park and Hu, 2010).

The aim of this study was to characterize the bacterial populations isolated in GSP agar from the water fraction, before and after RO treatment. These isolates, which could belong to the *Pseudomonas* genus, were assessed for their capacity to form biofilms *in vitro*. Additionally, their antibiotic resistance was evaluated, as this could increase their associated health risk. The isolates were biochemically characterized and identified using matrix-assisted laser desorption/ionization −time of flight mass spectrometry (MALDI-TOF MS). The effectiveness of the RO was also assessed by comparing the structure of the microbial communities isolated in the feed and permeate water.

2. Materials and methods

2.1. Sampling

The water samples were obtained from a drinking water treatment plant located in Spain (Sant Joan Despí, Barcelona) that treats 260,000–350,000 m^3/day. During the treatment process, water is pumped from a river and pre-treated by sequential treatments including: coagulation (aluminium salt)/flocculation (ferric chloride), sedimentation and sand filtration; this is followed by ultrafiltration using polyvinylidene fluoride membranes, then cartridge filtration and UV disinfection, to control for fouling, before it enters to the RO system. The RO system is comprised of 10 units, and each operating at a flow rate of 265 L/s. A unit consists of 158 tubes and each tube contains 7 RO membranes. The RO units involve a three-stage recirculation process which recovers between 85% and 90% of the incoming water. The RO membranes are made of polyamide/polysulfone casted onto polyester fabric. High-pressure pumping (8–16 bar) forces the water through the semipermeable membranes. Finally, a remineralisation with calcite filters and chlorination step is applied.

The water samples were collected before the water entered the RO units: feed water (F); and after the water passes through the semi-permeable membrane: permeate water (P). Two sampling campaigns were carried out in two consecutive years. Samples were taken in two different moments of the year, when the temperature of the water was at its annual highest (H) (between 20 °C and 24 °C) and when the temperature was at its lowest (L) (between 10 °C and 13 °C). Relevant water parameters such as temperature, pH, conductivity, turbidity and TOC (total organic carbon) were also determined. Volumes of feed water and permeate water between 100 and 1000 mL were filtered through 0.45 μm mixed cellulose ester filters. The filters were incubated on GSP agar plates (Merck Millipore, Germany) supplemented with Penicillin-G at 28 °C ± 2 °C for 2 days. GSP agar is a selective medium used for the detection of *Pseudomonas* and *Aeromonas* in water supplies (Kielwein, 1969). After incubation, a maximum of 25 isolates from each sample were selected from the filters and subcultured on Brain Heart Infusion agar (BHIA) (Laboratorios Conda, Spain).

2.2. Biochemical fingerprinting and population similarity

Fresh cultures were prepared on BHIA plates for 72 ± 2 h at 30 °C ± 2 °C for biochemical fingerprinting using the PhenePlate™ system (PhP system) (PhPlate Microplate Techniques AB, Sweden). These biochemical characterization microplates are based on a total of 48 tests which have been selected to provide a high level of discrimination of populations based on their phenotypic profile. Cell suspensions were prepared by harvesting these cultures in a suspending medium: 0.1% w/v proteose peptone and 0.011% w/v bromothymol blue with a solution of distilled water, according to the manufacturer's instructions. Aliquots of 150 μl were transferred into each well and the inoculated PhP system plates were incubated at 30 °C ± 2 °C. Growth in the wells was measured using an iEMS Reader MF (Labsystems, Finland) at 620 nm. Five readings were taken at 16 h, 40 h, 64 h, 88 h and 122 h. *P. aeruginosa* NCTC 10,332$^{(T)}$ was used as an internal control in all the experiments to confirm reproducibility. The biochemical profiles were calculated as previously described (Kühn et al., 1991) using the PhPWin® software (PhPlate Microplate Techniques AB, Sweden). On the basis of the results of the biochemical tests, isolates for each population were pooled to determine the biochemical phenotypes. Clusters were defined by isolates showing a similarity index equal to or higher than 0.950, using the unweighted pair group method with arithmetic mean (UPGMA). The isolates showing the highest mean similarity within a cluster and the lowest correlation with other clusters were selected as the representative isolates of phenotypic clusters for further characterization (Kühn et al., 1991): biofilm formation and antimicrobial susceptibility testing.

The structure and composition of the populations was analysed by cluster analysis on the basis of the PhP system profiles of the isolates. The similarity between populations in each sampling group was calculated using the coefficient of population similarity, Sp (Hunter and Gaston, 1988; Kühn et al., 1991). Thus, bacterial populations were considered similar when the Sp value was greater than 0.2. Calculation of the population similarity indexes was performed using the software PhPWin® software (PhPlate Microplate Techniques AB, Sweden).

2.3. MALDI-TOF MS spectrometry

The isolates were identified by applying mass spectrometry-based proteomics using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Germany). This method allows differentiation of bacteria by analysis of protein profiles. Briefly, intact bacteria were transferred from a culture grown on a BHIA plate (30 °C ± 2 °C for 24 ± 2 h) to a MALDI target plate and overlaid with a 1 μl solid organic matrix (saturated alpha-cian-4-hydroxycinamic acid-50% acetonitrile-2.5% trifluoroacetic acid). A pulsed UV laser (60 Hz) irradiated the crystallised sample; energy produced resulted in the disintegration of the crystals, and the ions released were accelerated in an electric field and measured using a Mircroflex II mass spectrometer (Bruker Daltonik GmbH, Germany). The protein spectra obtained with the spectrometer were processed with Bruker MALDI Biotyper v2.0 software (Bruker Daltonik GmbH, Germany) and compared with the Bruker spectra database. The results were categorized as follows: score <1.700, no reliable identification; score between 1.700 and 1.999, identification at genus level; and score ≥2.000, species identification.

2.4. 16S rRNA gene sequencing

The representative isolate of the phenotype groups which could not be identified by MALDI-TOF MS was genotypically

characterized by 16S rRNA gene sequencing. DNA extraction, amplification and 16S rRNA gene sequencing of each representative isolate was performed as previously described (Casanovas-Massana et al., 2010). The sequences were submitted for homology searches to the National Center for Biotechnology Information using BLAST (http://www.ncbi.nlm. nih.gov/Blast/) and deposited in GenBank. A cut-off at $\geq$ 97% similarity was used for isolate identification at the species level (Stackebrandt and Goebel, 1994).

2.5. Biofilm formation

Biofilm formation of the representative isolates of each PhP system group was evaluated using 96-well polystyrene cell-culture treated microtiter plates using the crystal violet staining method, as previously described (Stepanović et al., 2007). Overnight cultures of the representative isolates to be tested were prepared in TSB at 30 °C ± 2 °C. Afterwards, they were diluted in TSB so as to obtain an optical density of approximately 0.01 $OD_{600\ nm}$ units. A total of 200 μl was inoculated in each well, six replicates were performed and incubated statically in a wet chamber for 48 h at 30 °C ± 2 °C.

2.6. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the representative isolates of each PhP system group was determined using standard disc diffusion techniques, according to the recommendations of the Clinical Laboratory Standards Institute (National Committee for Clinical Laboratory Standards., 2014). Cultures were grown O/N overnight at 30 °C ± 2 °C in TSA and used to inoculate a saline suspension that was adjusted to 0.5 McFarland. Mueller-Hinton plates were inoculated, antibiotic discs were placed onto the surface of the inoculated agar plate. The discs used were Neo-Sensitabs (Rosco, Taastrup, Denmark): aztreonam (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg), gentamicin (10 μg), imipenem (10 μg), streptomycin (10 μg) and tetracycline (30 μg). The plates were incubated O/N at 30 °C ± 2 °C and the diameters of the inhibition zone measured.

3. Results and discussion

The feed and permeate water characteristics, in terms of temperature, conductivity, turbidity, pH and TOC are summarized in Table S1. Significant changes in the permeate water were observed in conductivity, turbidity and TOC, which decreased considerably. The pH of the permeate water was reduced by 1 unit after RO, since RO was removing alkaline mineral constituents. Nevertheless, no changes were reported in the temperature after RO in comparison with feed water. With regards to the sampling campaigns (1 and 2), the water parameters tested remained stable. The bacterial counts at high temperature were around 1 CFU 100 ml^{-1} in feed and permeate water. At low temperature the bacterial counts were 1.5 × 10^2 CFU 100 ml^{-1} and 1 CFU 100 ml^{-1} in feed and permeate water, respectively.

3.1. Population similarities

A total of 191 isolates were obtained (Table S1) and biochemically characterized using the PhP system: a total of 100 isolates when the water temperature was low (50 isolate at each point: feed and permeate water) and 91 when the temperature was high (46 from feed water; 45 from permeate water). The isolates were grouped into clusters according to the biochemical profiles obtained using the PhP system. A total of 158 out of the 191 isolates were grouped into 19 phenotypic groups.

The results of the population similarities are illustrated in Fig. 1. At high temperatures, the structure of the populations between campaigns showed considerable similarity (Fig. 1) for both sampling points in feed water, HF(1) and HF(2); and in permeate water, HP(1) and HP(2), respectively. This indicates that the water was colonized by some isolates sharing the same biochemical profiles when the temperature of the water was high. The Sp value of the isolates from feed water was 0.55, while for the isolates from permeate water it was 0.46; which supports the similarity of the populations. Phenotypic clustering of the feed water samples at high temperatures during both sampling campaigns showed clusters containing isolates from both campaigns (Fig. S1a). Consequently, the bacterial communities from the different sampling campaigns are indeed similar, displaying the same phenotypic profiles. Whereas, the structure between the populations of feed and permeate water in the same campaign was different. Isolates from feed and permeate water at high temperatures in the first sampling (Fig. S1b) formed separate clusters. Only two isolates were grouped into a cluster composed mainly of isolates from a different sampling point. Similar dendrograms were obtained with the other sampling points and campaigns (data not shown).

Isolates recovered from low temperature samples displayed different clustering in the two sampling campaigns. Isolates recovered from the samples collected in the first campaign had an Sp value of 0.52 between feed (LF(1)) and permeate (LP(1)) water, suggesting the same profiles dominated the populations on both sides of the RO (Fig. 1). Meanwhile, slightly less similarity was revealed in the low temperature isolates recovered from the second sampling campaign. Notably, the low temperature isolates from sample LF(2) were more similar to the isolates from the samples collected at high temperatures. According to the phenotypic profiles, the isolates from the different sampling campaigns at low temperatures but from the same point, feed water, formed different phenotypic clusters (Fig. S2a), in contrast to the results obtained at high temperatures (Fig. S1a). The isolates from the different sampling points (feed and permeate water) at low temperatures (Fig. S2b) shared some phenotypic profiles. Thus, the bacterial communities suspended in the water on the two sides of the RO membrane were phenotypically similar in the first campaign, whereas in the second campaign, the feed and permeate isolates did not share phenotypic profiles (data not shown).

The results obtained in the present study confirm that RO alters

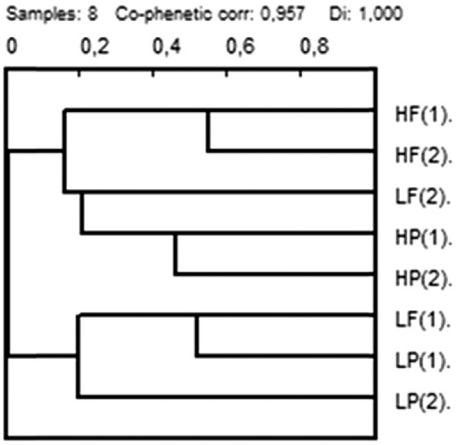

Fig. 1. Dendrogram of the similarity indexes (Sp) of the isolates suspended in feed and permeate water, using the UPGMA method. LF: feed water before RO at lower temperature. LP: permeate water after RO at lower temperature. HF: feed water before RO at higher temperature. HP: permeate water after RO at higher temperature. Sampling campaign is indicated in brackets.

the composition of the bacterial community capable of growing in GSP medium in water, since different communities were observed in the permeate water and the feed water. Although some phenotypic groups contained isolates from both sides of the RO system, it is not possible to establish the origin of those isolates, since RO membranes retain bacteria very effectively, and in general do not allow bacteria or viruses to pass through the membrane. However, the detection of microorganisms in permeate water was not unexpected, since the RO system and the pipes that connected it to the remineralisation tank are not a sterile system, and microorganisms could eventually recolonize the system. Moreover, the bacterial community in the permeate water must be adapted to selective pressures exerted by that water, with low nutrient levels, and according to the chemical parameters of the permeate water (Table S1).

3.2. Isolates identification

Identification of the isolates by MALDI-TOF MS enabled us to characterize the populations from the different sampling points (Fig. 2) at the genus level. The *Acidovorax* and *Pseudomonas* genera predominated among the 9 genera identified in the water samples studied. The *Pseudomonas* genus accounted for nearly 70% of the population and it was present in both seasons. However, the abundance of *Pseudomonas* at low water temperatures was substantially higher than at high temperatures. In fact, *Pseudomonas* was the major genus detected in all the low temperature samples except for LF(2), as indicated in the Sp dendrogram (Fig. 1); that sample being substantially different from the other low temperature samples. *Stenotrophomonas* isolates were detected mainly in the high temperature samples in feed water. Isolates belonging to the *Acidovorax* genus were mostly found in high temperature samples; in low temperature samples they were only detected in one sample, LF(2), which was closer to the permeate water samples at high temperatures, as shown in Fig. 1. The genera conforming the population with fewest isolations at high temperatures were *Achromobacter* (HP(1)), *Acinetobacter* (HF(1–2)), *Enterobacter* (HF(2)) and *Rhizobium* (HP(1)); while *Serratia* was only identified in one low temperature sample: LF(1). A large number of isolates which could not be characterized by MALDI-TOF MS were isolated from the feed water samples at high temperatures (HF(1) and HF(2)). This group represented more than 50% of the population and included two phenotypic groups that accounted for 60% of these isolates unidentified by MALDI-TOF MS. The 16S rRNA gene of the representative isolates of these two phenotypic groups were sequenced revealing that the isolates belonged to the *Ensifer adhaerens*. Sequences were deposited in GenBank under the accession numbers KU891984 and KU891985. According to a previous study, *Ensifer* spp. are not included in MALDI-TOF MS databases (Uhlik et al., 2011). Our results indicate that water samples collected at low temperatures had a different bacterial community from those detected in high temperature samples, as shown in the phenotypic clustering.

The bacterial structure in high temperature samples changed after the water passed through the RO. A preponderance of *Ensifer* and *Stenotrophomonas* was registered in water prior to RO, HF(1) and HF(2). In contrast, *Acidovorax* and *Pseudomonas* were the most abundant in the samples after RO: HP(1) and HP(2). This structure was repeated in both sampling campaigns. In contrast, *Pseudomonas* isolates predominated in feed and permeate water at low temperatures, with the exception of sample LF (2).

The MALDI-TOF MS results allowed us to identify some isolates belonging to *Pseudomonas* and *Stenotrophomonas* at the species level (Table S2). A total of 16 different species were detected among the *Pseudomonas* genus. *Pseudomonas* spp. are widespread throughout nature and have been isolated from many different water samples, such as drinking water (Bereschenko et al., 2008). As noted, some of the species were isolated only at a specific water temperature. As an example, isolates belonging to *Pseudomonas frederiksbergensis*, *Pseudomonas libanensis*, *Pseudomonas mandelii* and *Pseudomonas veronii* were detected at low temperatures. In contrast, isolates affiliated to *Pseudomonas nitroreducens* and *Pseudomonas putida* were recovered at high temperatures. *P. aeruginosa* was not detected in any of the samples analysed. In this respect, the water meets the quality standards and the bacterial community in the water does not constitute a health risk.

Although the selective GSP medium used was described as selecting *Pseudomonas* and *Aeromonas*, isolates identified belonging to other genera were isolated in the water samples before and after RO. This is in agreement with previous studies (Ribas et al., 2000), in which isolates belonging to *Comamonas, Burkholderia, Chryseomonas* and *Sphingomonas* were recovered, showing their capacity to grow on this selective medium.

The community structure at high temperature in feed water was

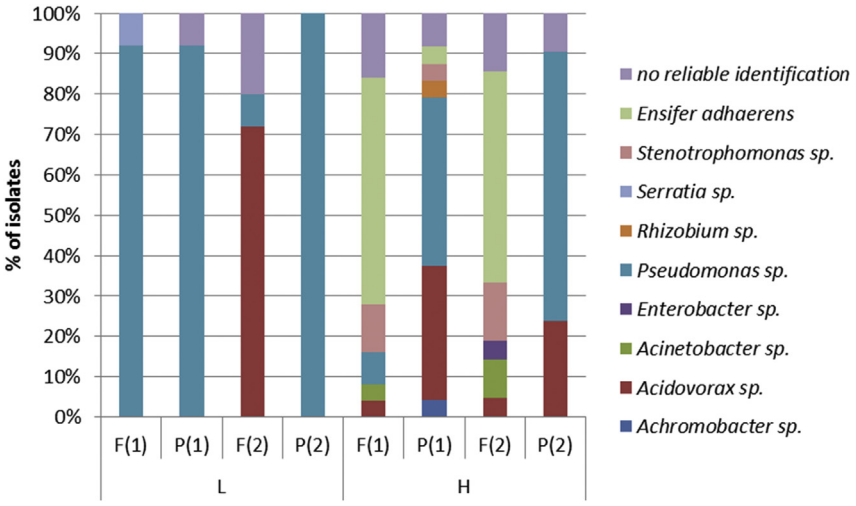

Fig. 2. Isolates identified by MALDI-TOF MS at the genus level showing the relative abundance in the microbial community suspended in water at the two sampling points, feed (F) and permeate (P) at different temperatures, low (L) and high (H). Sampling campaign is indicated in brackets. *Ensifer adhaerens* was identified by 16S rRNA gene sequencing.

Table 1
Antibiotic resistance of the representative isolates of the groups identified using the PhP system.

Isolate	MALDI-TOF MS identification	AZT	CAZ	CIP	CLR	CTX	GEN	IMI	SXT	STR	TET
HF(1).13	*E. adhaerens*[a]	R	R		R	I				R	
HF(1).16	*Sten. maltophilia*	R	R	I	R	R	R	R		R	R
HF(1).20	*E. adhaerens*[a]	R	R		R	I				R	
HP(1).22	*Acid. delafieldii*										
HF(2).6	*Acinetobacter* sp.	R	R		R	I					
LF(1).1	*P. veronii*	R	R		I	I			I		
LP(1).15	*P. libanensis*	R			R	I			R		
LP(1).7	*P. veronii*	R			I	R			R		
LF(1).22	*P. jessenii*	R			R	I			R		
LF(1).15	*P. frederiksbergensis*	R			R	R			R		
LP(1).12	*P. koreensis*	I	R		R	R			R		
HP(1).11	*P. putida*	I			R	R			R		I
HP(2).2	*P. putida*	I			R				R		I
HP(2).14	*P. nitroreducens*	R			I	I			R	R	
HP(2).24	*P. nitroreducens*	R			R	I			R	I	
LP(1).5	*P. proteolytica*	R			R	I			R		
LP(2).1	*P. mandelii*	R			R	R			R		
LP(2).17	*P. veronii*	R			I	R			R		
LP(2).14	*P. veronii*	R			I	R			R		

AZT, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CLR, chloramphenicol; CTX, cefotaxime; GEN, gentamicin; IMI, imipenem; SXT, trimethoprim + sulfamethoxazole; STR, streptomycin; TET, tetracycline. R, resistant; I, intermediate resistance.
[a] 16S rRNA gene sequencing identification.

more diverse than in the rest of the samples studied. One potential explanation for this change in the microbial community may be that a broader range of genera can develop at high temperatures (Ayache et al., 2013) than at low temperatures. Temperature plays an important role in the selection of the genera. Moreover, the chemical parameters of permeate water exert a pressure on the bacterial community, leading to a limited number of genera being capable of developing. Bacterial communities from water at low temperatures and permeate water are under both pressures; this could explain the similarity between feed water populations at low temperatures and permeate water all year round, which were dominated by isolates from the *Pseudomonas* and *Acidovorax* genus. In all cases, the permeate water, after RO treatment, meets the microbiological parameters for its intended use.

3.3. Biofilm formation

The spectrophotometric assay of biofilm formation performed on the representative isolates revealed that only four isolates produced biofilms *in vitro*. Isolate HF(1).16- *Stenotrophomonas maltophilia* was the best biofilm producer (OD_{570} = 0.728 ± 0.042), followed by isolate HF(1).13- *Ensifer adhaerens*, with an OD_{570} of 0.596 ± 0.024. Then HF(2).6-*Acinetobacter* sp. produced biofilm with an OD_{570} of 0.279 ± 0.014, and the lowest value was obtained for the isolate HP(2).2- *P. putida* (OD_{570} = 0.171 ± 0.016). These genera have also been reported to form biofilm or attach to biofilms from drinking water networks as reported below. Species belonging to the *Acinetobacter* genus have the capacity to adhere to surfaces and form biofilm (Bereschenko et al., 2008; Norton and LeChevallier, 2000; Simões et al., 2010). Most of the isolates in this study belonged to the *Pseudomonas* genus, and although not all of them could form biofilm *in vitro*, this genus has often been reported among biofilm from RO membranes (Bereschenko et al., 2010, 2008; Pang et al., 2005; Sadr Ghayeni et al., 1998). *E. adhaerens* and *S. maltophilia* have also been observed in biofilms from RO membranes (Ayache et al., 2013; Norton and LeChevallier, 2000), which is in agreement with the observed capacity for biofilm formation of the isolates of this study. Isolates with this capacity were isolated in both feed and permeate water, so they may be involved in biofilm formation and cause fouling over time.

3.4. Antimicrobial susceptibility testing

The isolates were classified, as shown in Table 1, as: susceptible, having intermediate resistance or resistant, according to the inhibition zone diameter of each antibiotic for *P. aeruginosa* (National Committee for Clinical Laboratory Standards, 2014; Pérez-Monrás et al., 2006; Vaz-Moreira et al., 2012). The most frequent antibiotic resistance (including intermediate resistance) profile was to aztreonam, chloramphenicol and cefotaxime, which was found in 95% of the isolates. Interestingly, only *Sten. maltophilia* was resistant to ciprofloxacin, gentamicin and imipenem. *Sten. maltophilia* and *P. putida* isolates showed resistance or intermediate resistance to tetracycline; while the other isolates presented susceptibility to this antibiotic. In contrast, a high level of intermediate resistance or full resistance to aztreonam and chloramphenicol was observed among the isolates tested. Moreover, only the *Pseudomonas* genus isolates were resistant to trimethoprim + sulfamethoxazole, which is in accordance with a previous study in which high percentages of resistance to a sulphonamide antibiotic class were revealed (Vaz-Moreira et al., 2012).

The *Acidovorax* isolate was susceptible to all the antibiotics tested. *Ensifer* isolates and the *Acinetobacter* isolate showed a nearly identical antibiotic resistance profile, with resistance to aztreonam, ceftazidime, chloramphenicol and cefotaxime. *E. adhaerens*, isolated from soil, has been reported to be resistant to chloramphenicol and streptomycin (Rogel et al., 2001). *Sten. maltophilia* was the isolate with the highest number of resistances, 9 (aztreonam, ceftazidime, ciprofloxacin, chloramphenicol, cefotaxime, gentamicin, imipenem, streptomycin and tetracycline) thereby showing considerable antibiotic multiresistance. In fact, clinical *S. maltophilia* isolates exhibit resistance to a broad class of antibiotics, although differences in the bacterial genome between clinical and environmental isolates have been reported (Brooke, 2012).

The most common resistance profile observed among *Pseudomonas* isolates was resistance to four antibiotics (aztreonam, chloramphenicol, cefotaxime and trimethoprim + sulfamethoxazole). In general, low antibiotic resistance was detected among *Pseudomonas* isolates, in agreement with a previous study in which the antibiotic resistance phenotypes of *Pseudomonas* from different drinking water sources were

evaluated (Vaz-Moreira et al., 2012). *Pseudomonas* isolates from drinking water were all susceptible to ciprofloxacin, gentamicin, imipenem and streptomycin according to Vaz-Moreira et al., 2012. In another study of *Pseudomonas* isolates from drinking water (Flores Ribeiro et al., 2014), the most common resistances were to aztreonam and trimethoprim + sulfamethoxazole, while less common resistance was observed to ceftazidime, ciprofloxacin and imipenem, whereas no gentamycin resistance was reported. A similar resistance pattern was observed in the present study for *Pseudomonas* sp. isolates. The study by Vaz-Moreira et al., 2012 demonstrated the importance of vertical resistance transmission; however, antibiotic resistance spread through horizontal gene transfer cannot be ruled out (Muniesa et al., 2013). Moreover, environmental *Pseudomonas* isolates have been shown to be more susceptible to antibiotics than clinical isolates (Ruiz et al., 2004).

4. Conclusions

Several *Pseudomonas* spp. but also other genera were isolated, but the communities suspended in the water column on the different sides of the RO membrane were different; this implies correct RO system performance and bacterial recolonization after RO. The communities at high temperatures were more diverse and different between raw and permeate water than at low temperatures. In any case, *P. aeruginosa* was not detected before or after RO treatment. Furthermore, some isolates had the capacity to form biofilm and presented resistance to some antibiotic in both raw and permeate water, which may contribute to future membrane fouling and antibiotic spread.

Conflict of interest

No conflict of interest declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2016.07.089.

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5.DISCUSSIÓ

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Els indicadors microbians de la contaminació fecal han estat útils al llarg dels anys pel control sanitari de la qualitat de l'aigua de consum, evitant així riscos sanitaris per a la població. Tot i així, l'aigua mineral embotellada i l'aigua de la xarxa de distribució no són aigües estèrils sinó que contenen una gran diversitat de microorganismes ambientals propis de cada tipus d'aigua. Actualment, la normativa vigent no exigeix el coneixement de la comunitat microbiana pròpia de cada tipus d'aigua. De fet, hi ha poca informació sobre aquestes comunitats de l'aigua, degut a la complexitat de l'estudi d'aquests ambients. Els requeriments necessaris pel seu creixement *in vitro* i la velocitat lenta de creixement en són alguns factors.

Aquesta tesi s'ha endinsat en l'estudi d'aquests ecosistemes com són les comunitats heteròtrofes de les aigües de consum, tant de l'aigua mineral natural envasada com l'aigua de xarxa de distribució durant el procés de potabilització a l'ETAP de Sant Joan Despí.

Aigua mineral natural

Les aigües minerals naturals són ecosistemes complexos des d'un punt de vista fenotípic i genètic. Les fonts i els aqüífers contenen una microbiota pròpia que les diferència entre elles, tal i com diversos estudis han reportat (Casanovas-Massana and Blanch, 2012; Loy et al., 2005; Rosenberg, 2003). Aquesta microbiota no potser modificada durant el procés d'envasament segons estableix la normativa. De fet, aquesta microbiota és el component natural de l'aigua que determina que sigui un producte viu i que evolucioni al llarg de la seva vida comercial, tal i com es va determinar a l'article 1. Els recomptes d'heteròtrofs en placa obtinguts no van ser constants durant l'envelliment de l'aigua dins l'ampolla, sinó que fluctuaven, com els valors de la diversitat de Simpson. De fet, les comunitats heteròtrofes de l'aigua mineral embotellada analitzada van mostrar períodes de creixement i decreixement. La diversitat de Simpson també va presentar comportaments diferents en funció de la marca i del lot al llarg de la vida comercial de l'aigua. De fet, les fluctuacions dels heteròtrofs en placa van coincidir amb les de la diversitat a les aigües A2, B1 i B2. Així els increments en la concentració de microorganismes heteròtrofs van correspondre amb increments de la diversitat.

Únicament es va observar una disminució de la concentració d'heteròtrofs i de la diversitat després d'un any. A més, la concentració d'heteròtrofs no va ser menyspreable prop de la data de consum preferent, entre 2 i 3 $\log_{10}$ UFC/ml d'heteròtrofs en placa. Així doncs,

transcorregut un any des de l'envasat encara trobem una comunitat bacteriana viable i cultivable que ha persistit a les condicions oligotròfiques de l'aigua dins l'ampolla.

També es van observar diferències significatives entre les concentracions obtingudes entre els lots de la mateixa marca en els diferents temps estudiats. Concretament, es van observar diferències significatives ($p<0,0001$) (Anàlisi de la variància corregit amb HSD de Tukey) entre els recomptes d'heteròtrofs en placa a 125 i 365 dies dels lots de l'aigua A i a 55 i 365 dies de l'aigua B. Aquests resultats suggereixen que el comportament dels heteròtrofs és diferent a cada lot, tot i estar envasat a la mateixa època de l'any en anys consecutius.

L'índex de diversitat de Simpson va oscil·lar entre 0,57-0,87 i 0,35-0,97 en les aigües A i B respectivament al llarg de l'estudi. En general, en aquest estudi, els valors de diversitat que es van obtenir van ser baixos. En canvi, en un estudi previ on s'aïllaven microorganismes heteròtrofs de l'aigua de les fonts i dels aqüífers abans de l'envasat van obtenir valors de diversitat alts, fet que indicava que hi havia una gran diversitat fenotípica en aquelles aigües (Casanovas-Massana and Blanch, 2012). Així doncs, després de l'envasat de l'aigua, la diversitat podria disminuir i la comunitat que inicialment a l'aqüífer podia ser més diversa, passaria a ser dominada per pocs fenotips, tal i com es demostra en la caracterització genotípica.

Les oscil·lacions dels recomptes d'heteròtrofs en placa com de la diversitat preveien que la composició de la comunitat variaria amb el temps, fet que queda corroborat en els dendrogrames dels coeficients de similitud poblacional. Aquests dendrogrames mostren com les comunitats d'heteròtrofs aïllades al mateix lot eren diferents en els tres temps estudiats. Això indica que cada ampolla era un micro-ecosistema aïllat de l'exterior que va canviar de manera diferent amb el temps, malgrat totes les ampolles del mateix lot es van envasar al mateix dia i es van mantenir en les mateixes condicions d'emmagatzematge.

La identificació de les soques representatives mitjançant la seqüenciació del gen ARNr 16S va confirmar les diferències entre les comunitats bacterianes entre les diferents marques i al llarg de l'emmagatzematge tal i com s'havia intuït amb el coeficient de similitud poblacional. Això concorda amb estudis previs on s'havien observat diferències entre la comunitat heteròtrofa entre diferents aigües minerals naturals procedents de diferents orígens (Hunter, 1993; Loy et al., 2005; Mavridou, 1992; Venieri et al., 2006). Segons diversos estudis, els microorganismes més freqüentment aïllats a l'aigua mineral

pertanyen a les 3 classes (*Alfaproteobacteria, Betaproteoacteria* i *Gammaproteobacteria)* del fílum *Proteobacteria* (Falcone-Dias et al., 2012; Loy et al., 2005; Otterholt and Charnock, 2011; Rosenberg, 2003). La classe *Alfaproteobacteria* va predominar entre les soques aïllades en les dues aigües estudiades. De fet, en l'aigua A només es van detectar soques que pertanyien a la classe *Alfaproteobacteria* i en l'aigua B a més d'*Alfaproteobacteria* també es van detectar alguna soca de la classe *Gammaproteobacteria* (gèneres *Pseudomonas* i *Pseudoxanthomonas*), com també la presència d'*Actinobacteria* (gènere *Mycobacterium*). La majoria dels gèneres que es van identificar en les aigües minerals A i B havien estat prèviament aïllats en aigües minerals. Tanmateix no es té constància que els gèneres *Sphingopyxis, Blastomonas* i *Pseudoxanthomonas* hagin estat descrits en aigües minerals però sí en d'altres matrius d'aigua com les aigües termals, l'aigua de distribució i l'aigua de mar o en el sòl (Baik et al., 2013; Hwang et al., 2012; Li et al., 2014; Revetta et al., 2010; Tokajian et al., 2005; Yoo et al., 2007; Yoon and Oh, 2005; Yoon et al., 2005).

A més a més, la composició de les comunitats heteròtrofes va ser diferent entre els dos lots de la mateixa marca. Només compartien un gènere cada una, *Brevundimonas* i *Pseudomonas*, en les aigües A i B respectivament, entre els dos lots de la mateixa marca i temps estudiats. No obstant això, no es van detectar al llarg de tot el període d'estudi, la qual cosa no implica que no hi fossin presents en menor quantitat i que no s'haguessin detectat. Aquestes diferències entre lots no s'esperaven, ja que les aigües minerals naturals per definició no han de presentar variacions significatives en les seves propietats fisicoquímiques, de manera que els ecosistemes microbians associats a una aigua en particular haurien de ser força constants. Aquestes diferències detectades entre lots es podrien atribuir, per exemple, a que les comunitats no es distribueixen uniformement en els estrats de l'aqüífer i les variacions en el punt de captació podrien mobilitzar diferents microambients dins el mateix aqüífer i ser responsables d'aquestes diferències. No obstant això, no podem excloure la possibilitat que les comunitats van ser similars just després de l'embotellat i després es van diferenciar, ja que aquest estudi no es va centrar en les primeres etapes. Alternativament, els procés d'embotellament pot introduir canvis en les condicions ambientals, com l'augment de la temperatura de l'aigua, i aquestes tinguin un efecte en el desenvolupament de la comunitat heterotròfica.

Atenent als resultats del primer article, en que no es podia explicar el comportament de les comunitats heteròtrofes amb els mètodes dependents de cultiu i per tant no es podien utilitzar com a traçadors de l'aigua, en el segon article relacionat amb l'aigua mineral natural s'empraren, a més, mètodes no dependents de cultiu, per a valorar la comunitat

bacteriana com a traçadora de l'aigua mineral. A més, en aquest article s'estudià la mateixa aigua mineral envasada en diferent material d'ampolla (PET i vidre) per observar possibles variacions en l'estructura de la comunitat. L'estudi es realitzà durant 3 mesos, que equival aproximadament al temps que les ampolles es troben en el mercat abans de ser adquirides pels consumidors.

Un dia després de l'embotellament, els recomptes d'heteròtrofs van ser molt baixos <50 UFC/ml per a les 3 marques d'aigua mineral natural (a excepció de l'aigua A envasada en PET, $3x10^2$ UFC/ml), en canvi, el total de cèl·lules presents a l'aigua va ser de l'ordre de 3 log_{10}cèl·lules/ml. Una gran part d'aquesta comunitat es trobava en un estat de no viabilitat, ja que les cèl·lules viables només representaven un 10% del nombre total de cèl·lules. Possiblement el procés d'embotellament pot afectar la viabilitat de les cèl·lules o que els microorganismes en l'aqüífer es trobin en un estat de latència. No obstant, una setmana després de l'envasat, les cèl·lules viables van incrementar fins a representar aproximadament el 50% del total de cèl·lules. De fet, en les ampolles de vidre, les cèl·lules viables van assolir valors majors que en les respectives ampolles de PET, tot i que l'increment va ser més lent en el temps. Respecte els recomptes del nombre total de cèl·lules, aquests van seguir una tendència paral·lela a les cèl·lules viables, és a dir, un increment entre les dues primeres setmanes i es van mantenir estables durant els 3 mesos de l'estudi. De fet, com en altres estudis, els valors de cèl·lules totals que es van obtenir van ser similars entre $1x10^4$ i $1x10^5$ cèl·lules/ml (Defives et al., 1999; Loy et al., 2005) tant en les ampolles de PET com vidre.

Els recomptes d'heteròtrofs en placa van augmentar considerablement, fins a 3 i 4 unitats log_{10} en 15-20 dies segons la marca. Aquest increment es va produir primer a les aigües envasades en ampolles de vidre. Després de l'augment de les cèl·lules cultivables, que totes les ampolles van experimentar, va anar precedit d'una disminució. A partir d'aquest moment, els recomptes d'heteròtrofs van oscil·lar sense seguir cap tendència tant per marca com per material. Per tant, la comunitat heteròtrofa cultivable tal i com es va observar en el primer estudi en temps més llunyans de la data d'envasat com en el segon estudi just després de l'envasat i en dos tipus de materials d'ampolla diferents, els recomptes d'heteròtrofs fluctuen al llarg del període comercial de les ampolles d'aigua mineral natural. Potser una part de la comunitat bacteriana podria adherir-se a les parets de l'ampolla, per la qual cosa, les fluctuacions en els recomptes d'heteròtrofs podrien ser degudes per aquest fenomen. Tot i no haver estudiat l'existència de cèl·lules adherides a la paret, en el supòsit que els recomptes d'heteròtrofs poguessin haver estat alterats degut aquest fenomen, possiblement també s'haguessin observat patrons similars en els valors

totals de cèl·lules i en les cèl·lules viables. En canvi, els valors totals de cèl·lules i els de viables van ser constants al llarg dels 3 mesos.

No es van observar diferències en referencia al nombre de cèl·lules totals i viables entre els materials de l'ampolla a partir del dia 21i fins a finals de l'estudi, quan els recomptes de cèl·lules totals i viables es van estabilitatzar, no obstant, les empremtes moleculars de cada marca i material analitzats van ser característiques. L'empremta molecular obtinguda mitjançant la tècnica de la DGGE va ser semblant per a cada marca d'aigua mineral en les dues ampolles (PET i vidre) a les 24 hores després de l'embotellament, tot i que a la marca A, certes diferències en el bandeig s'intuïen. Les empremtes moleculars després de l'envasat contenien un nombre major de bandes que va disminuir després d'una o dues setmanes i es va mantenir estable fins al final de l'experiment. Només una setmana després de l'envasat, la comunitat bacteriana en 3 aigües minerals; aigua mineral A i B envasada en PET i aigua mineral C en vidre, va experimentar un canvi que es va reflectir en un perfil de bandes diferent. De fet, el perfil de bandes de la DGGE va ser constant durant els 3 següents mesos analitzats en aquestes aigües que es van diferenciar ràpidament de l'empremta molecular inicial. En aquestes empremtes moleculars predominen clarament 2-3 bandes que es mantenien constants. Per tant, la comunitat present a l'aigua mineral es va adaptar ràpidament a les condicions ambientals de l'interior de l'ampolla i aquesta comunitat es va mantenir estable en el temps. En canvi, les marques d'aigua que es van diferenciar respecte de l'inicial més tard, els perfils de bandes van fluctuar durant els 3 mesos, és a dir, la comunitat bacteriana no va ser constant.

Cada marca d'aigua mineral va mantenir la seva pròpia empremta molecular fins al final de l'experiment i atès que eren diferents entre elles tal i com es va observar en el dendrograma i en el PCA, no es pot excloure la possibilitat d'identificar algun traçador bacterià propi de cada aigua. Tot i que, degut a la complexitat dels perfils obtinguts, possiblement seria necessari l'elecció d'un conjunt de traçadors per a poder distingir les marques d'aigua. A més, les ampolles seleccionades a l'atzar d'altres lots van presentar bandes comunes amb les identificades al lot analitzat durant 3 mesos, fet que dóna suport a la possilibtat de definir marcadors moleculars per a la traçabilitat de l'aigua.

El material de l'envàs podria afectar el desenvolupament de la comunitat bacteriana tal i com queda plasmat en les empremtes moleculars. Malgrat l'aigua es va envasar en els dos materials en el mateix dia i per tant, podem assumir que la comunitat bacteriana a era igual, la comunitat va evolucionar diferent. Les bandes de la DGGE que es van detectar en tots els temps analitzats per a cada marca d'aigua van ser diferents en funció del material

de l'ampolla. Les bandes que es van detectar en tots els temps estudiats en la marca d'aigua mineral A, tant en PET com en vidre, cada una pertanyia a una classe de *Proteobacteria* diferent i també al fílum *Actinobacteria.* Només aquesta última es va detectar en tots els temps estudiats en les dues ampolles (PET i vidre). En canvi, en la marca d'aigua mineral B i C (ambdós materials), les bandes constants durant els 3 mesos estudiats es van associar a la classe *Betaproteobacteria.* No obstant, aquestes bandes no van ser les mateixes en les ampolles de PET i vidre de la mateixa aigua mineral. Els fragments de les bandes amplificats tenien una longitud de només 180 pb, per aquest motiu, les identificacions obtingudes per similitud respecte les seqüències dipositades en les bases de dades s'han de prendre amb cautela. Cal destacar, que moltes de les bandes seqüenciades es van associar a bacteris no cultivats.

En estudis previs s'ha proposat que els compostos orgànics que es podrien alliberar del material plàstic de les ampolles podrien afavorir el creixement bacterià (Bischofberger et al., 1990) o que colors foscos de l'envàs podrien protegir a la comunitat bacteriana de la radiació solar i així, el creixement esdevingués superior durant la seva vida comercial, segons un estudi (Mavridou, 1992). En el present estudi només els recomptes de viables i d'heteròtrofs de les aigües envasades amb vidre van assolir concentracions majors que en plàstic. Així mateix les ampolles de la marca B que presentaven un envàs de color fosc, no es van observar diferències respecte les altres aigua envasades en ampolles incolores.

En conclusió, en els 2 estudis realitzats es demostra que les aigües minerals naturals envasades són uns microsistemes on la comunitat bacteriana canvia amb el temps, no només en els primers dies després de l'envasat sinó fins a l'any. La successió de les comunitats microbianes que tenen lloc en les ampolles pot ser impulsada per la diferent capacitat dels microorganismes de sobreviure en un entorn oligotròfic i tancat o com a resultat de la reactivació de cèl·lules en estat de latència inicialment presents a l'aigua mineral, que explicarien els increments que es produeixen després de l'envasat. De fet, algunes espècies poden créixer a despenses de la matèria orgànica a partir d'espècies lisades, d'aquesta manera les espècies bacterianes s'anirien succeint (Leclerc and Moreau, 2002). Aquests dos estudis realitzats dins aquesta tesi doctoral sobre l'aigua mineral natural envasada indiquen que tot i ser un sistema tancat, és un sistema molt complex.

Per altra banda, la caracterització i la traçabilitat de la microbiota natural de l'aigua embotellada pot ser un objectiu estratègic per a les empreses del sector per tal de posicionar-se en el lideratge del sector.

Aigua de xarxa de distribució

L'estudi de les comunitats bacterianes en les ETAPs podria utilitzar-se per millorar la gestió de les plantes de tractament d'aigua. Aquesta informació permetria entendre l'efecte dels diferents tractaments sobre la comunitat bacteriana i per a detectar ràpidament errors en el sistema i/o potencials patògens que puguin representar un risc per a la salut pública i així poder realitzar les operacions correctores oportunes. No obstant això, és necessari el desenvolupament de noves tècniques analítiques que es puguin aplicar en rutina per tal de monitoritzar les comunitats en els propis laboratoris de les plantes de tractament.

Els avenços en la biologia molecular han permès superar els problemes associats al cultiu dels microorganismes de les aigües de consum i obtenir una imatge taxonòmica acurada de la diversitat. Algunes d'aquestes tècniques són l'amplificació de fragments variables del gens del ARNr 16S mitjançant PCR i seguit de la DGGE i les tècniques de seqüenciació massiva. Encara que, aquestes tècniques són complexes i l'obtenció dels resultats no és instantani sinó que requereix uns dies pel procediment experimental i l'anàlisi dels resultats. A més, és necessari personal especialitzat per realitzar el procediment experimental, el processament de les dades i la interpretació dels resultats. En conseqüència, aquestes tècniques no són aplicables en els laboratoris de rutina i per tant, no són útils per monitoritzar freqüentment les comunitat microbianes presents en l'aigua per tal detectar canvis o situacions anòmales.

L'article 3 d'aquesta tesi és el primer treball que s'ha valorat la utilització de la tecnologia emergent MALDI-TOF MS per a l'ús en rutina per la identificació de soques aïllades en una ETAP. Aquesta tècnica es va comparar amb el sistema PhenePlate™, que ha estat àmpliament utilitzat en el grup de recerca per a l'estudi de comunitats microbianes (Casanovas-Massana and Blanch, 2013, 2012; Vilanova et al., 2002) i amb les galeries API®, que és la metodologia freqüentment utilitzada en els laboratoris de rutina. El percentatge de soques identificades amb el MALDI-TOF MS a nivell d'espècie (39%) va ser molt semblant al percentatge obtingut amb les galeries API® (43%). A més, un 26% van ser identificades a nivell de gènere amb MALDI-TOF MS. No obstant això, un 35% de les soques no van poder ser identificades amb el MALDI-TOF MS i un 57% amb API®. Les soques que segons la seqüenciació del gen ARNr 16S pertanyien als gèneres *Bergeyella, Cloacibacterium, Hydrogenophaga* i *Lysinibacillus* no van poder ser identificades per MALDI-TOF MS, de fet la base de dades d'espectres proteics de la casa comercial que es va emprar en aquest estudi, contenia un nombre limitat d'espectres d'aquestes espècies.

Concretament 2 espectres de les espècies del gènere *Hydrogenophaga*, d'un total de 10 espècies descrites i 3 espectres de 21 espècies del gènere *Lysinibacillus.* En canvi, del gènere *Bergeyella* només s'ha descrit una espècie i aquesta està introduïda a la base de dades. És necessari estudiar aquesta soca aïllada per si és una espècie nova d'aquest gènere. En canvi, el gènere *Cloacibacterium* no està inclòs. En el cas de les soques que no van poder ser identificades amb API® va ser degut a que la base de dades API® no conté informació sobre les espècies dels gèneres *Acidovorax, Cloacibacterium, Flavobacterium, Hydrogenophaga* i *Janthinobacterium.* En canvi, per *Bergeyella, Lysinibacillus* i *Shewanella* només conté informació d'una o dues espècies d'aquests gèneres. En 6 soques aïllades, la combinació dels resultats de les proves bioquímiques de les galeries API® va generar una identificació errònia. Aquest és el cas de les soques que pertanyien a *Acidovorax, Bergeyella, Flavobacterium* i *Hydrogenophaga* que van ser identificades erròniament com a B*revundimonas, Ochrobactrum* i *Sphingomonas,* amb més d'un 90% de precisió de similitud*.* En canvi, MALDI-TOF MS va ser més acurat a les identificacions, ja que si l'espècie de la soca aïllada no estava disponible a la base de dades no va ser identificada. Possiblement, les 20 proves bioquímiques de les galeries API 20NE no són suficients com per discernir entre el gran nombre d'espècies que es poden aïllar a l'aigua. No tots els grups fenotípics del sistema PhenePlate™ van contenir un únic espectre de MALDI-TOF MS, tot i així, en tots els grups, l'espectre majoritari obtingut va coincidir amb la identificació del gens ARNr 16S. De fet, el 80% de les soques van obtenir el mateix resultat a la identificació comparant els resultats obtinguts entre les tècniques MALDI-TOF MS i la seqüenciació del gen ARNr 16S a partir dels grups fenotípics del PhenePlate™.

La caracterització de les soques aïllades amb MALDI-TOF MS va permetre realitzar una anàlisi instantània de la comunitat microbiana present al llarg del tractament de l'aigua (article 4). Aquesta caracterització va mostrar un canvi dels microorganismes al llarg del procés, inicialment, l'aigua d'entrada de la planta i en els primers estadis del tractament predominaven els microorganismes Gram negatius fins el clar predomini dels microorganismes Gram positiu a l'aigua tractada. Aquest canvi es va observar tant a l'estació de l'hivern com a l'estiu.

Les comunitats presents en els diversos punts del tractament com també a l'aigua potabilitzada depenen de la microbiota present a l'aigua d'origen i de d'ordre dels tractaments aplicats durant el procés de potabilització, ja que cada tractament afecta diferentment a les comunitats microbianes. Així doncs, és difícil comparar les comunitats microbianes amb altres plantes de tractament d'aigua, ja que els processos aplicats són diferents de l'ETAP estudiada en aquest treball, com també les característiques de l'aigua

d'origen. Els microorganismes que pertanyien a la classe *Betaproteobacteria* dins el fílum *Proteobacteria* van ser aïllats amb major freqüència. Els microorganismes de la classe *Betaproteobacteria* es caracteritzen per tenir un creixement ràpid i preferir ambients amb concentracions de nutrients elevades (Newton et al., 2011). A més, la classe *Betaproteobacteria* és particularment sensible a la cloració (Kormas et al., 2010; Poitelon et al., 2010; Williams et al., 2004), el que podria explicar la seva abundància en les primeres etapes, on l'aigua té una concentració més alta de nutrients i la disminució progressiva al llarg del tractament de l'aigua.

La comunitat bacteriana al llarg del procés va canviar tal i com s'observa en el dendrograma de les similituds poblacions calculades a partir del sistema PhenePlate™. Únicament les comunitats de l'efluent del filtre de sorra, ultrafiltració i osmosi inversa van ser semblants ja que compartien un gran nombre de soques dels gèneres *Acidovorax, Acinetobacter* i *Chryseobacterium.* Els diversos tractaments aplicats van ser una barrera per la majoria dels microorganismes. Principalment la cloració va tenir un paper important en la determinació de la composició bacteriana final, doncs va provocar un canvi dràstic en la composició, tal i com altres estudis previs també han demostrat (Eichler et al., 2006; Poitelon et al., 2010).

A més a més, les comunitats van ser diferents en les dues estacions analitzades. En aquest estudi, alguns gèneres es van identificar només a l'hivern com *Flavobacterium, Janthinobacterium, Kocuria*, *Pantoea*, *Serratia* and *Shewanella*. En canvi, els gèneres *Arthrobacter, Chryseobacterium*, *Comamonas, Cupriavidus, Dickeya, Elizabethkingia*, *Lysinibacillus*, *Massilia*, *Microbacterium, Sphingopyxis* i *Wautersiella* van ser identificats només a l'estiu. Les comunitats eren complexes, doncs van obtenir valors de diversitat poblacional elevats (Di>0,800) tant a l'hivern com a l'estiu fet que indica que no hi havia cap fenotip majoritari en els diferents punts del procés, sinó que hi havia una gran varietat de perfils bioquímics diferents. Els valors de diversitat van disminuir a mesura que l'aigua superava els diferents tractaments. La població de l'aigua tractada que estava formada per soques del gènere *Bacillus* no va poder ser fenotipada mitjançant el sistema PhenePlate™. El procés d'inoculació d'aquest sistema no és adequat per aquest tipus de soques amb una textura greixosa i també perquè les soques aïllades no van fermentar els sucres de les plaques PhP-48 que es van utilitzar.

La caracterització de les comunitats presents en l'ETAP mitjançant MALDI-TOF MS es pot obtenir amb poc més de 24-72 hores, que depèn bàsicament del temps necessari per l'aïllament de les soques, ja que la identificació amb MALDI-TOF MS d'una soca bacteriana

es realitza amb menys de 10 min. Algunes estimacions calculen que el cost d'identificació d'una soca amb MALDI-TOF MS és aproximadament 1,5€, relativament més econòmic que una galeria API®, que el seu cost oscil·la entre 4,6-6,0€. De manera que l'aplicació de MALDI-TOF MS permet reduir el temps de resposta ja que l'obtenció dels resultats és ràpida, millorar l'exactitud dels mètodes d'identificació fenotípics convencionals i reduir el cost del material fungible com també el temps de dedicació dels analistes.

Tot i els avantatges que ofereix aquesta tècnica, el percentatge de soques que no van poder ser identificades per transferència directa va ser molt elevat en alguns punts, principalment en les comunitats de l'efluent del filtre de sorra i de l'osmosi inversa. En el cas de la comunitat de l'aigua tractada, on un gran nombre dels aïllats pertanyien al gènere *Bacillus*, el percentatge d'identificats possiblement milloraria amb l'extracció de proteïnes de les soques ja que la paret bacteriana dels microorganismes Gram positius pot dificultar la ionització de les proteïnes, però el temps emprat per l'anàlisi incrementaria.

Aquesta tècnica permet agrupar per similitud els espectres proteics no identificats, doncs permet reduir el nombre de soques que serà necessari realitzar una caracterització més complerta en el cas que es vulgui identificar la soca i introduir-la en la base de dades pròpia. A més, els espectres proteics de totes aquelles soques no identificades queden registrats, i els espectres proteics de les noves soques aïllades es poden comprar per similitud amb els anteriors. De manera que, es pot registrar la freqüència d'aïllament de les soques, tot i no conèixer l'espècie.

Les soques aïllades en els controls rutinaris de les comunitat heteròtrofes es poden identificar ràpidament per MALDI-TOF MS, d'aquesta manera es pot obtenir una caracterització complerta de les comunitats al llarg de l'any en una ETAP i aplicar aquesta informació en millores en la gestió. D'altra banda, també es poden seleccionar aquelles soques anòmales obtingudes en els controls rutinaris o que tinguin cert interès per l'empresa gestora, i identificar-les ràpidament per tal de descartar la presència de patògens o soques que interfereixin en el procés de potabilització.

En els estudis realitzats s'evidencia la falta d'una base de dades suficientment àmplia pels microorganismes ambientals, dificultant l'aplicabilitat de MALDI-TOF MS i la potencialitat d'aquesta tècnica. Les biblioteques disponibles actualment estan dirigides a la identificació de bacteris procedents de mostres d'origen clíniques. Disposar d'una base de dades validades per microorganismes de l'aigua redundaria en benefici del control microbiològic

rutinari que realitzen les empreses encarregades de la gestió de l'aigua. De fet, aquesta tècnica ofereix la possibilitat de caracteritzar la comunitat heteròtrofa habitual d'una ETAP i una capacitat de resposta ràpida a les empreses gestores de l'aigua de distribució en cas de detectar anomalies.

La tecnologia de filtració per membrana d'osmosi inversa (OI) s'ha implantat com a part del procés de tractament de l'aigua en les ETAPs per tal de proporcionar aigua de major qualitat. Tot i així, es plantegen certs inconvenients que poden afectar l'operativitat i la qualitat sanitària de l'aigua com és el deteriorament de les membranes amb el temps produït per la formació de biofilm sobre les membranes. La formació de biofilm requereix major consum d'energia de les bombes d'aigua d'alimentació i provoca la disminució del flux d'aigua osmotitzada i la reducció de la qualitat de l'aigua. Fins i tot, pot provocar l'obstrucció de la membrana, com a resultat, suposa pèrdues econòmiques en el sector de la gestió de l'aigua. En conseqüència, el coneixement d'aquestes comunitats és interessant des d'un punt de vista econòmic, però també sanitari, doncs els biofilms són uns sistemes que poden albergar microorganismes patògens oportunistes com *Pseudomonas aeruginosa*. Per aquest motiu, en l'estudi que tenia per objectiu comparar les comunitats microbianes planctòniques de l'aigua d'alimentació i l'aigua osmotitzada, es va emprar un medi selectiu per l'aïllament diferencial de *Pseudomonas* i *Aeromonas,* el medi GSP (article 5). Altres bacteris que no pertanyien a aquests dos gèneres es van recuperar en les mostres d'aigua. En estudis previs, Ribas i col·laboradors, també van aïllar amb el medi GSP soques que pertanyien a altres gèneres com *Comamonas, Burkholderia, Chryseomonas* i *Sphingomonas*. En conseqüència, es fa palès que altres microorganismes tenen la capacitat de créixer en aquest medi selectiu, GSP. D'altra banda, l'espècie *P. aeruginosa*, que podia posar en risc la qualitat de l'aigua de consum, no es va detectar en cap de les mostres analitzades, per tant l'aigua complia amb els estàndards de qualitat pel seu ús previst regulats a la normativa.

Les membranes d'osmosi inversa retenen els microorganismes per exclusió de mida de porus, tot i així a l'aigua osmotitzada es va detectar presència microbiana. L'aigua osmotitzada, malgrat ser un aigua ultrapura i un ambient extrem per la majoria dels microorganismes, en aquest treball es van obtenir recomptes de microorganismes en el medi GSP de 1 UFC/ml a temperatura de l'aigua alta i baixa. Tot i que no està clar que la presència de microorganismes a l'aigua osmotitzada es relacioni amb el pas dels

microorganismes a través de la membrana, tal i com estudis anteriors han reportat (Park and Hu, 2010).

Els resultats de l'estructura i la composició de les comunitats obtingudes confirmen que les membranes d'OI alteren la composició de la comunitat bacteriana, ja que les comunitat microbianes que es van observar a l'aigua d'alimentació abans d'entrar a l'OI i a l'aigua osmotitzada després del seu pas per la membrana eren diferents. Tot i així, alguns grups fenotípics compartien soques amb un mateix perfil bioquímic aïllades a banda i banda del sistema d'osmosi inversa. Aquest fet, no implica que aquests aïllats tinguessin el mateix origen, sinó que presentaven un comportament bioquímic similar. De fet, no és possible establir l'origen dels aïllats de l'aigua osmotitzada, amb la informació que s'ha recollit en aquest estudi. Eventualment, els microorganismes podrien recolonitzar el sistema de membranes, ja que les connexions dels bastidors de les membranes amb el tanc de remineralització no són uns sistemes estèrils. Potser alguns microorganismes han estat capaços d'instaurar-se en les membranes o en les canonades de l'aigua osmotitzada durant els processos de manteniment, substitucions de membranes o en algun cas travessar el sistema en cas de ruptura de les membranes. Aquests motius podrien explicar que certs microorganismes puguin ser aïllats en un aigua que aparentment no hauria de contenir bacteris.

El pas de l'aigua a través de l'OI produeix canvis en la població que es tradueixen en una disminució del nombre d'espècies, tal i com es va observar en les mostres de temperatura alta. En canvi, en les mostres de l'aigua a temperatura baixa, no es va observar el mateix patró en els dos mostreigs realitzats. En el primer mostreig, l'aigua d'alimentació i l'aigua osmotitzada es podien considerar semblants segons els dendrograma dels coeficients de similitud. En el segon mostreig, la comunitat de l'aigua d'alimentació era més pròxima a les comunitats de temperatura alta. La presència d'espècies del gènere *Acidovorax* a les mostres de l'aigua d'alimentació a temperatura baixa i a les mostres de l'aigua osmotitzada a temperatura alta, podrien explicar aquesta similitud entre elles. D'altra banda, l'aigua osmotitzada en el segon mostreig realitzat a temperatura baixa era més semblant a les mostres del primer mostreig a temperatura baixa, on les espècies del gènere *Pseudomonas* predominaven a les comunitats. Les dues mostres de l'aigua d'alimentació a temperatura alta van ser les més diverses en comparació amb les altres mostres estudiades. La temperatura és un factor de selecció molt important, a temperatura més elevada es poden desenvolupar més gèneres (Ayache et al., 2013). D'altra banda, la comunitat bacteriana de l'aigua osmotitzada ha d'estar adaptada a unes pressions selectives exercides pels paràmetres fisicoquímics de l'aigua, per tant, un nombre limitat d'espècies poden

desenvolupar-se. Això podria explicar que les comunitats després de l'OI siguin menys diverses. Així que, les comunitats de l'aigua osmotitzada com l'aigua d'alimentació a temperatura baixa es troben sota pressions, siguin les característiques fisicoquímiques de l'aigua o la temperatura, per aquest motiu es podria explicar la similitud entre aquests dos tipus de comunitats.

Tot i que les espècies aïllades en aquest sistema de membranes d'osmosi inversa no constitueixin un risc per a la salut, algunes d'elles tenen l'habilitat de formar biofilm i totes elles presenten algun tipus de resistència a antibiòtics.

Un total de 4 soques testades van presentar capacitat de formació de biofilm. Aquestes soques corresponien als gèneres *Acinetobacter, Ensifer, Pseudomonas* i *Stenotrophomonas,* els quals han estat prèviament aïllats i relacionats en biofilms associats en aigües de distribució (Ayache et al., 2013; Bereschenko et al., 2010, 2008; Norton and LeChevallier, 2000; Pang et al., 2005; Sadr Ghayeni et al., 1998; Simões et al., 2010). L'espècie *Ensifer adhaerens* i algunes espècies del gènere *Acinetobacter* i *Pseudomonas* són productors d'acil-homoserina lactona, molècules senyal per la comunicació intraespecífica dels sistemes de *quorum sensing* (Huang et al., 2012; Subhadra et al., 2016) que poden intervenir en la formació de biofilm. També, la motilitat *swarning* del gènere *Pseudomonas* pot afavorir la colonització de les superfícies (Harshey, 2003). *Stenotrophomonas maltophilia* és considerat un patogen oportunista emergent, aïllat en pacients o en ambients hospitalaris però també aïllat habitualment al medi ambient, en l'aigua de riu, sistemes de potabilització d'aigua i la rizosfera de les plantes entre altres. *Sten. maltophilia* representava una petita proporció de la comunitat de l'aigua d'alimentació a temperatura alta i aquesta soca va ser la major productora de biofilm. De fet, aquesta espècie s'ha aïllat en biofilms en sistemes d'aigua potable i s'ha descrit que en aquests sistemes es pot trobar formant microcèl·lules de 0,1-0,2 μm (Brooke, 2012; Norton and LeChevallier, 2000; Penna et al., 2002). A més, les soques amb capacitat de formar biofilm es van aïllar tant en l'aigua d'alimentació com en l'aigua osmotitzada, per tant existeix la possibilitat que aquestes soques puguin estar implicades en la formació de biofilm a les dues bandes d'aquest sistema al llarg del temps. No podem descartar que les altres soques que no van desenvolupar biofilm *in vitro*, puguin fer-ho en el propi sistema on les interaccions i sinèrgies entre microorganismes són més complexes.

En general, entre les soques aïllades de *Pseudomonas* es va detectar una baixa resistència a antibiòtics, una mitjana de 4 resistències als 10 antibiòtics avaluats per a cada soca aïllada.

Les resistències més freqüents van ser als antibiòtics aztreonam, cloramfenicol, cefotaxima i trimetoprima+sulfametoxazol. Aquests resultats concorden amb un estudi previ en el qual es van avaluar els fenotips de resistència a antibiòtics de *Pseudomonas* de soques procedents de diversos punts d'una planta de tractament d'aigua potable que també van trobar una baixa resistència entre les soques testades (Vaz-Moreira et al., 2012). De fet, les soques aïllades del gènere *Pseudomonas* tenien un patró similar amb estudis previs (Vaz-Moreira et al., 2012) ja que les soques de *Pseudomonas* van mostrar susceptibilitat a la ciprofloxacina, gentamicina, imipenem i estreptomicina. En un altre estudi, les soques de *Pseudomonas* d'una planta de tractament d'aigua potable (Flores Ribeiro et al., 2014) van mostrar resistència a l'antibiòtic trimetoprima+sulfametoxazol, tal i com es va detectar en les nostres soques aïllades. La resistència a trimetoprima+sulfametoxazol pot ser habitual en soques del gènere *Pseudomonas,* de fet, en un estudi es va posar en manifest la baixa capacitat inhibidora d'aquest antibiòtic contra *P. aeruginosa* (Köhler et al., 1996). Les soques aïllades en el present estudi van ser susceptibles als antibiòtics ceftazidima, ciprofloxacina i imipenem, en canvi, en l'estudi de Flores Ribeiro detectaven algunes resistències a aquests antibiòtics. Tot i que les soques aïllades en aquest sistema presentaven resistència a certs antibiòtics i aquest fet pot comportar un risc per a la salut, les soques aïllades en el medi ambient de *Pseudomonas* a diferència de les clíniques han demostrat ser més susceptibles als antibiòtics (Ruiz et al., 2004). *Sten. maltophilia* va mostrar resistència a la majoria dels antibiòtics testats. Encara que, els aïllats clínics de *Sten. maltophilia* presenten generalment multiresistències a una àmplia classe d'antibiòtics, s'han descrit nombroses diferències entre el genoma bacterià dels aïllats clínics i dels ambientals (Brooke, 2012). És difícil establir un patró de resistència en funció de la localització de les soques aïllades, doncs només es van testar les soques representatives dels grups fenotípics. Tanmateix amb els patrons obtinguts, la resistència a antibiòtics està més relacionada amb l'espècie que no en el punt d'aïllament (abans o després de l'OI) ja que no hi ha diferències en el nombre de resistències. Això concordaria amb l'estudi de Vaz-Moreira i col·laboradors que suggereix la importància de la transmissió vertical de gens de resistència perquè el perfil de resistències està més lligat a les espècies que no en els punts d'aïllament. No obstant, la transferència horitzontal de gens de resistència a antibiòtics a través dels bacteriòfags en les aigües no es pot menysprear (Muniesa et al., 2013).

6.CONCLUSIONS

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Aigua mineral natural

- L'aigua mineral constitueix un ecosistema microbià complex que canvia al llarg del temps. No es poden generalitzar els canvis de les comunitats heteròtrofes cultivables entre diferents marques d'aigües però tampoc entre lots de la mateixa marca, que es comporten independentment, tot i que hi ha una disminució del nombre d'heteròtrofs en placa i de la diversitat després d'un any.
- L'estructura de la comunitat bacteriana experimenta un canvi en les dues primeres setmanes després de l'envasat, caracteritzat per un augment de les cèl·lules totals i les viables en les dues primeres setmanes. L'increment inicial que té lloc després de l'envasat podria estar influenciat pel material de l'ampolla, tot i que, una vegada assolides les concentracions màximes, no s'observen diferències significatives en els recomptes entre diferents materials de l'ampolla.
- Algunes de les marques d'aigua mineral estudiades tenen una empremta molecular característica, la qual cosa suggereix la possibilitat de definir un marcador molecular per a la seva traçabilitat.

Aigua de xarxa de distribució

- La tecnologia MALDI-TOF MS és útil per a la identificació de les soques aïllades en una ETAP, per la seva rapidesa, simplicitat i robustesa, per tant és una tecnologia que pot ser implementada en rutina.
- La incorporació de soques ambientals en les bases de dades de MALDI-TOF MS permetria ampliar el potencial que ofereix aquesta tecnologia, actualment en són limitades.
- La capacitat d'identificació de MALDI-TOF MS és igual o superior a altres tècniques que s'utilitzen habitualment.
- L'acumulació de dades sobre les comunitats heteròtrofes d'una ETAP permet entendre la dinàmica de les poblacions al llarg de l'any i millorar la gestió del sistema de potabilització.
- Les poblacions microbianes planctòniques de l'aigua d'alimentació de l'OI i l'aigua osmotitzada presenten una composició i estructura diferent. Això suggereix un

correcte funcionament del sistema d'OI i una recolonització bacteriana després d'OI.

- *P. aeruginosa* no es detecta abans ni després de la filtració per OI, per tant, l'aigua compleix els paràmetres microbiològics per l'aigua de consum humà.
- Algunes soques aïllades tenen l'habilitat de formar biofilm *in vitro* i presenten resistències a certs antibiòtics, per tant la presència d'aquestes espècies pot estar relacionar amb la formació de biofilm i per tant el posterior deteriorament de les membranes d'OI i la dispersió de resistències a antibiòtics.

7.BIBLIOGRAFIA

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8.APÈNDIX

8.1. Apèndix 1- Càlcul diversitat de Simpson i similitud poblacional

Càlcul de la diversitat de Simpson i la similitud poblacional a partir de les dades obtingudes amb el sistema PhenePlate™.

La diversitat de la població bacteriana associada a cada aigua es calcula mitjançant l'Índex de diversitat de Simpson (Di) (Hunter and Gaston, 1988). L'índex es calcula segons:

$$D = 1 - \sum_{j=1}^{S} \frac{n_j(n_j - 1)}{N(N-1)}$$

N és el nombre de soques totals existents a la població i n_j el nombre de soques del mateix grup fenotípic.

La diversitat de Simpson oscil·la entre 0-1. La Di serà elevada (pròxima a 1) en el cas que les soques aïllades no comparteixen perfils bioquímics, és a dir, siguin diferents entre sí. En canvi, valors propera a 0, si tots els aïllats presenten el mateix perfil bioquímic.

El coeficient de similitud poblacional (Sp) indica la proporció de soques aïllades que són idèntiques entre dues mostres comparades (Hunter and Gaston, 1988; Kühn et al., 1991), segons la fórmula:

$$S_p = \left(S_x + S_y\right)/2$$

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:

$$S_x = \sum qx_i/N_x$$

$$S_y = \sum qy_i/N_y$$

$$qx_i = Px_i/Py_i$$

$$qy_i = Py_i/Px_i$$

$$N_x = nombre\ de\ soques\ de\ la\ mostra\ x$$

$$N_y = nombre\ de\ soques\ de\ la\ mostra\ y$$

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

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.

El coeficient de similitud poblacional depèn de la proporció de soques aïllades que siguin iguals entre les dues poblacions comparades, és a dir que comparteixin perfils bioquímics. Quan Sp pren valors baixos, indica que les poblacions són diferents entre elles. En canvi, si les poblacions comparteixen grups fenotípics, Sp serà major. Sp oscil·la entre valors de 0 a 1. Es considera que dues poblacions són idèntiques des d'un punt de vista fenotípic, quan s'obtenen coeficients de 0,2, ja que en rèpliques d'una mateixa mostra s'obtenen valors de Sp propers a 0,5.

A partir de la matriu dels coeficients Sp obtinguda al comparar les poblacions es pot representar gràficament la distància entre les poblacions en un dendrograma seguint el mètode UPGMA. Així s'obté un dendrograma que millor representi la similitud entre les poblacions analitzades.

8.2. Apèndix 2- Altres publicacions

Figuerola, B., **Sala-Comorera, L.**, Angulo-Preckler, C., Vázquez, J., Montes, M.J., García-Aljaro, C., Mercadé, E., Blanch, A.R., Avila, C., 2014. Antimicrobial activity of Antarctic bryozoans: An ecological perspective with potential for clinical applications. Mar. Environ. Res. 101C, 52–59. doi:10.1016/j.marenvres.2014.09.001

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Casanovas-Massana, A., **Sala-Comorera, L.**, Blanch, A.R., 2014. Quantification of tetracycline and chloramphenicol resistance in digestive tracts of bulls and piglets fed with Toyocerin(®), a feed additive containing *Bacillus toyonensis* spores. Vet. Microbiol. 173, 59–65. doi:10.1016/j.vetmic.2014.07.005

Reprinted from Veterinary microbiology, vol. 173, Arnau Casanovas-Massana, Laura Sala-Comorera, Anicet R. Blanch, Quantification of tetracycline and chloramphenicol resistance in digestive tracts of bulls and piglets fed with Toyocerin(®), a feed additive containing Bacillus toyonensis spores, 59-65, Copyright (2014), with permission from Elsevier.

García-Aljaro, C., Díaz-Pena, R., Casanovas-Massana, A., **Sala-Comorera, L.**, Blanch, A.R. Environmental detection and isolation of strains affiliated to *Bacillus toyonensis*, a species with probiotic representants. Està actualment sotmès a la revista *The Journal of General and Applied Microbiology.*

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Antimicrobial activity of Antarctic bryozoans: An ecological perspective with potential for clinical applications

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ABSTRACT

The antimicrobial activity of Antarctic bryozoans and the ecological functions of the chemical compounds involved remain largely unknown. To determine the significant ecological and applied antimicrobial effects, 16 ether and 16 butanol extracts obtained from 13 different bryozoan species were tested against six Antarctic (including *Psychrobacter luti*, *Shewanella livingstonensis* and 4 new isolated strains) and two bacterial strains from culture collections (*Escherichia coli* and *Bacillus cereus*). Results from the bioassays reveal that all ether extracts exhibited antimicrobial activity against some bacteria. Only one butanol extract produced inhibition, indicating that antimicrobial compounds are mainly lipophilic. Ether extracts of the genus *Camptoplites* inhibited the majority of bacterial strains, thus indicating a broad-spectrum of antimicrobial activity. Moreover, most ether extracts presented activities against bacterial strains from culture collections, suggesting the potential use of these extracts as antimicrobial drugs against pathogenic bacteria.

1. Introduction

The Antarctic continental shelves, with a relatively stable environment below the limit of ice scour and anchor ice, are characterized by the presence of a high diversity and abundance of sessile suspension feeders (Dayton et al., 1974). This stability leads to the sessile suspension feeder communities being mainly influenced by biotic interactions, favouring the evolution of chemical defences (Avila et al., 2008; McClintock et al., 2010). Among these benthic organisms, bryozoans are an important component of Antarctic biodiversity, with a total number of species estimated at over 390, with new species still being discovered (Kuklinski and Barnes, 2009; Hayward and Winston, 2011; De Broyer et al., 2011; Figuerola et al., 2013a). Antarctic bryozoans are often characterized by having circumpolar distributions and broad bathymetric ranges (Hayward, 1995). In particular, cheilostome bryozoans, the most successful living order of bryozoans, have developed a wide range of physical (e.g. avicularia or spines) and chemical mechanisms (natural products) used for roles, such as the defence against predators and prevention of settlement of epibionts fouling (Al-Ogily and Knight Jones, 1977; Winston, 1986, 1991; Walls et al., 1993; Lindquist, 1996; Iyengar and Harvell, 2002; Lopanik et al., 2006; Sharp et al., 2007; Carter et al., 2010; Figuerola et al., 2013b, 2014). Although thirty-five natural products have been isolated from cold-water (temperatures near zero degrees Celsius) bryozoans, none of these reports cite Antarctic species (Lebar et al., 2007). In fact, the absence of epibionts on the surface of many bryozoans is normally thought to be an evidence for the presence of these defences (Wahl, 1989; Shellenberger and Ross, 1998; Krug, 2006).

For instance, fouling is a common life-history trait in marine organisms, initiated by bacterial colonization (first stages) and, subsequently, by the settlement of unicellular and multicellular epibionts (Wahl, 1989). As marine organisms are constantly exposed to high concentrations of bacteria (Jenkins et al., 1998), some antimicrobial activity may be useful in limiting later successional fouling stages (i.e. settlement of epibionts) through prevention of "surface conditioning" by early stage bacteria (Wahl,

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1989). In the case of filter-feeding organisms that feed on bacteria, like bryozoans, surface colonization by microorganisms, including opportunistic pathogens, is favoured by their concentration from the water column during the feeding process (e.g. Winston, 1977; Bergquist, 1978). In fact, bryozoans often host communities of microorganisms and small invertebrates within their colony structures (Peters et al., 2003; Carter, 2008). In contrast, other microorganisms are inhibited by antimicrobial secondary metabolites produced by some bryozoan species, such as *Bugula dentata* Lamouroux, 1816, and *Flustra foliacea* Linnaeus, 1758 (Matsunaga et al., 1986; Wright, 1984).

Microbial biodiversity in Antarctic waters is also thought to be high but is still poorly explored (around $0.37 \cdot 10^6$ cells per ml; Zdanowski, 1995; Bej et al., 2010). In the present study, the bryozoans might potentially be in contact with the Antarctic bacterial strains tested, as diverse Antarctic bacterial strains of the genera *Bacillus* Cohn 1872, and *Micrococcus* Cohn 1872, have been found in sediment samples collected from the sea adjacent to King George Island, an island close to our study area (Zhou et al., 2013). Likewise, the genus *Paracoccus* Davis 1969 has been isolated from diverse Antarctic regions (e.g. Michaud et al., 2004; Heindl et al., 2012). In particular, *Bacillus aquimaris* Yoon et al., 2003, *Shewanella livingstonensis* Bozal et al., 2002, and *Oceanobacillus* sp. Lu et al., 2002, have all been isolated from Antarctic sponges in the Weddell and Ross Seas (Xin et al., 2011; Papaleo et al., 2012). Moreover, several species of the genus *Psychrobacter* Juni and Heym 1986 are widespread in Antarctic environments and some of them have been isolated from Antarctic sea ice and krill (Bowman et al., 1997; Denner et al., 2001). Interestingly, this genus has also been found associated with internal tissues of an ascidian collected in the Indian Ocean (Romanenko et al., 2002). With regard to bacteria isolated from bryozoan species belonging to the genus *Bacillus*, *Micrococcus*, *Psychrobacter* and *Shewanella* MacDonell and Colwell 1986 have been found them associated with the common boreal bryozoan species *F. foliacea* in the North Sea (Pukall et al., 2001). Additionally, species belonging to the genus *Bacillus*, *Paracoccus*, *Psychrobacter* and *Shewanella* have been isolated from the cosmopolitan bryozoan *Membranipora membranacea* Linnaeus, 1767 (Heindl et al., 2012). *Shewanella* has also been found associated with other bryozoan species from the North and Baltic Seas (Peters et al., 2003; Heindl et al., 2010). Singularly, this genus is considered an opportunistic pathogen of humans (e.g. Brink et al., 1995) and aquatic animals (Aguirre et al., 1994).

Regarding pathogenic bacteria, the evolution of antibiotic-resistant bacteria led to the search for potential antimicrobial agents from unexplored marine natural areas, such as Antarctica, which is considered a potential reserve of novel active compounds (Lebar et al., 2007; Avila et al., 2008). In particular, compounds from some bryozoans have demonstrated to present pharmacological properties, such as antitumour activity (e.g. de Vries and Beart, 1995). The active secondary metabolites from bryozoans could potentially be used as antimicrobial drugs against common bacteria, such as *Bacillus cereus* Frankland and Frankland 1887, and *Escherichia coli* (Migula 1895), which were tested in this study. On the one hand, *Bacillus* species are ubiquitous and diverse both in the terrestrial and marine ecosystems (Oguntoyinbo, 2007); *B. cereus* has been associated with fresh and pasteurised food products due to their ability to generate heat-resistant spores under adverse environmental conditions and it has been recognised as a food poisoning causative agent linked to food borne emetic and diarrhoeal syndromes (Ghelardi et al., 2002). On the other hand, some *E. coli* strains have been associated with food borne illness caused by consumption of raw or undercooked meat (Fischer et al., 2007).

To our knowledge, there is little information about the ecological and applied prospective roles of secondary metabolites as antimicrobials in Antarctic or any other bryozoans. Considering that the global bryozoan species richness is around 5869 spp and that now we know that diverse Antarctic species possess active compounds (e.g. Figuerola et al., 2012b; Bock and Gordon, 2013; Taboada et al., 2013; Figuerola et al., 2013b, 2014), there is room for more secondary metabolites as antimicrobials to be discovered. The aim of the present study is to assess the role and the potential pharmacological interest of bryozoan-derived natural products by investigating the antimicrobial activity of 16-ether and 16-butanol extracts from Antarctic bryozoans against a diverse array of sympatric bacterial populations and bacterial strains from culture collections.

2. Material and methods

2.1. Collection of bryozoan samples and identification

Sixteen abundant Antarctic bryozoan colonies (13 species) were collected in the eastern Weddell Sea (Antarctica) between 273.6 m and 351.6 m depth during the ANT XXI/2 (November 2003–January 2004) cruise on board the R/V Polarstern (Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany) using an Agassiz Trawl and a Bottom Trawl (Tables 1 and 2). These species show wide bathymetric ranges in their distributions in the Eastern Weddell Sea (Figuerola et al., 2012a). Bryozoan species offering a high diversity of potential

Table 1
Samples and data from the stations in the Weddell Sea.

Species	Latitude (S)	Longitude (W)	Depth (m)
Bostrychopora dentata Waters, 1904	70° 57.00′	10° 31.61′	284.4
Camptoplites angustus (1) Kluge, 1914	70° 50.75′	10° 28.01′	281.2
Camptoplites angustus (2) Kluge, 1914	70° 50.78′	10° 28.51′	273.6
Camptoplites bicornis Busk, 1884	70° 56.83′	10° 32.61′	338
Camptoplites tricornis (1) Waters, 1904	70° 56.67′	10° 32.05′	302.4
Camptoplites tricornis (2) Waters, 1904	70° 57.33′	10° 33.86′	351.6
Dakariella dabrowni Rogick, 1956d	70° 57.00′	10° 33.02′	332.8
Isoschizoporella secunda Hayward and Taylor, 1984	71° 06.44′	11° 27.76′	277.2
Isosecuriflustra tenuis Kluge, 1914	70° 52.75′	10° 51.24′	294.8
Klugella echinata Kluge, 1914	70° 56.83′	10° 32.61′	338
Melicerita obliqua Thornely, 1924	71° 06.44′	11° 27.76′	277.2
Nematoflustra flagellata Waters, 1904	70° 56.42′	10° 31.61′	284.4
Notoplites drygalskii (1) Kluge, 1914	70° 57.11′	10° 33.32′	337.2
Notoplites drygalskii (2) Kluge, 1914	71° 04.30′	11° 33.92′	308.8
Smittina antarctica Waters, 1904	71° 06.44′	11° 27.76′	277.2
Systenopora contracta Waters, 1904	71° 06.44′	11° 27.76′	277.2

Table 2
Distribution of bryozoans in the South Shetland Islands and Antarctic Peninsula. Additional data came from the website SCAR-MarBin, Barnes et al. (2008) (1), López-Fé and García Gómez (2000) (2), López-Fé (2004) (3) and Moyano (2005) (4).

Species	Deception Island	Greenwich Island	King George Island	Elephant Island	Antarctic Peninsula
Bostrychopora dentata				X[4]	X[4]
Camptoplites angustus	X	X	X	X[4]	
Camptoplites bicornis	X[1]	X	X	X[4]	X[3]
Camptoplites tricornis		X	X	X[4]	X[3]
Dakariella dabrowni					X
Isoschizoporella secunda			X[2]	X[4]	X[3]
Isosecuriflustra tenuis	X[1]	X		X[4]	X
Melicerita obliqua	X			X[4]	X[4]
Nematoflustra flagellata	X	X	X[2]	X[4]	X[4]
Notoplites drygalskii	X[1]		X[2]	X[4]	X[4]
Smittina antarctica		X		X[4]	X[4]
Systenopora contracta				X[4]	X[4]

microhabitats for bacteria species with different levels of calcification, from lightly calcified (e.g. *Nematoflustra flagellata* (Waters, 1904)) to highly calcified (e.g. *Melicerita obliqua* (Thornely, 1924)), and with several kinds of morphological structures (e.g. spines, avicularia…) were selected. At time of collection, the selected bryozoan colonies were sorted on deck, photographed, and a voucher sample of each colony was fixed in 70% ethanol for further taxonomical identification at the Faculty of Biology (University of Barcelona). All colonies were conserved at −20 °C prior to further examination. Most bryozoan species studied here have been reported to be widely distributed in Antarctica (Hayward, 1995) and specifically in the South Shetland Islands (Table 2). Of all the species studied, *Klugella echinata* (Kluge, 1914) is the only species recorded outside the region of the Antarctic Peninsula.

2.2. Chemical extractions of bryozoans

All bryozoan voucher samples were exhaustively extracted with acetone and sequentially partitioned into ether and butanol extracts. A water residue was also obtained and kept for further studies. All fractionation steps were repeated 3 times, except for the butanol fraction, which was done only once. Organic solvents were evaporated using a rotary evaporator. Ether (comprising the most apolar, lipophilic metabolites) and butanol (polar, hydrophilic metabolites) extracts were dissolved in a minimum volume of the extraction solvent and used for bioassays at natural concentrations.

2.3. Antarctic bacteria isolation

Two bacterial strains were isolated from Deception Island (South Shetland Is.); one from a stone (BAC03 and BAC02.1), one from sediment (BAC02.2) and one from the sponge species *Haliclona* sp. Grant, 1836 (BAC84) during two voyages: ACTIQUIM-3 (January–February 2012) and ACTIQUIM-4 (January–February 2013). Bacteria isolated from the stone and the sponge were collected by swabbing the surface with a sterile cotton-wool swab which was then transferred into sterile plastic tubes. Bacteria isolated from the sediment were collected directly using sterile plastic tubes. A 1 ml aliquot of all samples was added to Difco™ marine broth 2216 (Difco Laboratories, Sparks, MD, USA), left for 24 h at 18–20 °C, and subsequently cultured on Difco™ marine agar plates. The resulting bacterial extracts were isolated, and the strain exhibiting the fastest growth was chosen for the assays. A subsample of the selected strain was frozen at −20 °C and shipped to the University of Barcelona for further identification. These bacterial strains were maintained on 100% marine agar slopes at 4 °C and 15% (v/v) glycerol (870 g/l).

Bacterial strains from King George and Livingston Islands were isolated from seawater during a Spanish scientific expedition (austral Summer of 1987–1988). Strains of *Shewanella livingstonensis* (CECT 5933^T) were isolated from seawater in Johnson's Dock (Livingston Island, South Shetland Islands). Strains of *Psychrobacter luti* Bozal et al., 2003, (CECT 5885^T) were isolated from mud collected in Admiralty Bay (King George Island, South Shetland Islands), at the bottom of a glacier which is covered by water during high tide. These bacterial strains were maintained at the laboratory of the Faculty of Pharmacy (University of Barcelona) on TSA slopes at 4 °C and at −20 °C in 50% (v/v) glycerol (Bozal et al., 2002, 2003).

2.4. Bacterial strains and culture media

E. coli ATCC 700078 WG5 strain and *B. cereus* CECT 4014 strain were cultured on nutrient agar (NA) (Merck) at 37 °C for 18–24 h. Antarctic bacteria isolates from King George and Livingston Islands (*P. luti* and *S. livingstonensis*) were grown at 15 °C for 48 h on TSA. Antarctic bacteria isolates from Deception Island (strains BAC02.1, BAC02.2, BAC03 and BAC84) were grown at 20 °C for 48 h in marine agar 6 (Difco Laboratories, Sparks, MD, USA). Fresh cultures were used for antimicrobial testing.

2.5. Genotypic identification of Antarctic strains

DNA from Antarctic bacteria was isolated using a Wizard® Genomic DNA Purification Kit (Promega, Spain) following the manufacturer's instructions. The 16S rRNA gene was PCR amplified using universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). Reaction volumes of 50 μl were set up in the following manner: a final concentration of 1 × PCR Buffer, 2.5 U Taq polymerase, 200 μM of each deoxynucleoside triphosphate, 0.4 μM of universal bacterial primers and 2 μl of template DNA. PCR conditions included an initial denaturation step (94 °C, 5 min), 35 cycles of denaturation step (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1.5 min) and a final extension step (72 °C, 7 min). After agarose (1.5%) gel separation and ethidium bromide staining, PCR amplicons were checked under UV transillumination. PCR purification was done using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Alcobendas). PCR products were subsequently sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems, Alcobendas) and an ABI PRISM 3700 DNA Sequencer (Applied Biosystems, Alcobendas), following the manufacturer's instructions. The sequences attained were aligned with the BioEdit® program. The BLAST tool from NCBI was used to determine sequence similarity.

16S rRNA gene sequences reported in the paper have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/), with their corresponding accession numbers (see Table 3).

Table 3
Preliminary classification of cultured Antarctic bacteria isolated from Deception Island (South Shetland Is.).

Isolated from	Growth medium	Strain code	Accession number	The nearest type strain	GenBank acc. no	Identity (%)
Sediment	Marine broth	BAC02.1	KJ402360	*Micrococcus* sp.	EF175876.1	100%
Sediment	Marine broth	BAC02.2	KJ402361	*Bacillus aquimaris*	JF411285.1	100%
Sponge	Marine broth	BAC84	KJ402363	*Oceanobacillus* sp.	JN039424.1	100%
Stone	Marine broth	BAC03	KJ402362	*Paracoccus* sp.	GQ199613.1	98%

2.6. Antimicrobial testing

The agar disk diffusion method (Acar, 1980, M2-A11 Performance Standards for Antimicrobial Disk NCLS 2012) was used to test antimicrobial activity of extracts. A bacterial suspension from isolated colonies was prepared in saline buffer to achieve turbidity equivalent to 0.5 Mcfarland Standard. Using sterile cotton swabs, each microorganism was inoculated in triplicateon (i) the surface of Mueller–Hinton agar (Oxoid) (for the bacteria from the culture collection and Antarctic bacterial strains (*P. luti* and *S. livingstonensis*)) and (ii) Marine Agar (Difco) for the Antarctic bacterial strain from Deception Island. Paper antimicrobial assay disks (6 mm, BBL Microbiology Systems 31039, Cockeysville, MD, USA), were prepared by adding 20 μL of extract solutions (extract resuspended in the solvent) and evaporated to dryness. Disks were placed on the surface of inoculated plates (agar diffusion method). The volume added saturates the filter disk (Mahon et al., 2003). Each test consisted of one disc without any additive (negative control), one disc with a known antibiotic as positive control (chloramphenicol), one disc impregnated with solvent as the negative control (*methanol*), and one disc impregnated with the extract from the selected bryozoan at natural concentration in the organism. The inoculated plates were incubated at 37 °C for 18–24 h for *E. coli* and *B. cereus*, at 15 °C for at least 48 for *P. luti* and *S. livingstonensis* and at 20 °C for 48 for Antarctic bacteria from Deception Island. *E. coli* and *B. cereus* were also used as quality control strains for antimicrobial susceptibility testing with known responses to chloramphenicol. The radius of the inhibition zone (without disc) was measured. Levels of activity were categorized following the criterion of Lippert et al. (2003): no effect (0), weak inhibition (0–1 mm), moderate inhibition (>1–3 mm), strong inhibition (>3–7 mm) and very strong inhibition (>7–15 mm). These categories, however, have to be considered with caution since the diffusion rate of some antimicrobial compounds could perhaps affect the size of the halii too. For example, temperature, nutrients and growth conditions could modify expression, cell physiology and efflux transporter systems (e.g. Martín-Rodríguez et al., 2014).

3. Results

3.1. Identification of bacterial strains from Deception Island

Based on 16S rRNA sequence similarity, isolated strains BAC02.1, BAC02.2, BAC03 and BAC84 were most closely related to *Micrococcus* sp., *B. aquimaris*, *Paracoccus* sp. and *Oceanobacillus* sp. respectively (Table 3).

3.2. Antimicrobial tests

Antimicrobial activity was tested in 16 ether and 16 butanol extracts obtained from 16 bryozoan samples belonging to 13 common and abundant Antarctic bryozoan species in the proximities of the South Shetland Islands, the Antarctic Peninsula, and the Weddell Sea (Tables 1 and 2). The bioassays revealed that all the ether extracts tested at natural concentrations exhibited antimicrobial activity against some bacteria (Table 4). The collected bryozoans demonstrated most antimicrobial activity against the Antarctic bacteria. Antimicrobial activity was detected against seven bacteria in the ether extracts of *Camptoplites bicornis* (Busk, 1884) and *Camptoplites tricornis* (Waters, 1904) (sample 2). In particular, *C. bicornis* produced the strongest inhibition (halii of inhibition between 7 and 15 mm against strain BAC02.1.). Ether extracts from *C. bicornis*, *C. tricornis* (sample 1) and (2), *K. echinata*, *M. obliqua*, *Notoplites drygalskii* (Kluge, 1914) (2) and *Smittina antarctica* (Waters, 1904) were effective against both bacterial strains from culture collections. *Dakariella dabrowni* (Rogick, 1962) showed antimicrobial activity against only one bacterial strain (*E. coli*). Five species tested here exhibited activity against the bacterium isolated from the sympatric sponge *Haliclona* sp. (strain BAC84). The majority of the ether extracts displayed halii of inhibition between 0 and 3 mm. Only one butanol extract of *Systenopora contracta* Waters, 1904, displayed activity against one Antarctic bacterial strain BAC02.2 (halii of inhibition between 1 and 3 mm). Strain BAC03 was resistant to antimicrobial agents of all bryozoan species. In contrast, strain BAC02.2 and strain BAC02.1 showed less resistance.

Fourteen ether extracts belonging to 12 bryozoan species were active against Antarctic bacteria strain BAC02.1 and 11 were active against strain BAC02.2 (Table 4). Only two ether extracts from *C. bicornis* and *C. tricornis* (2) out of 16 were active against Antarctic bacterial strains from the seawater of King George and Livingston Islands. Intra and interspecific variability was detected in the genus *Camptoplites* and intraspecific variability in *N. drygalskii*.

More than 87% of the bryozoan samples studied were active against at least one of the two bacterial strains from culture collections used, which were also the quality control strains for antimicrobial susceptibility testing (Table 4). Seven ether extracts displayed antimicrobial activity against both *E. coli* and *B. cereus*. Five ether extracts were effective only against *E. coli*, while two ether extracts were effective only against *B. cereus*. *C. bicornis* and *K. echinata* showed the highest activity level against the Gram-positive bacteria *B. cereus*. Most extracts were more effective against the Gram-negative bacteria *E. coli*. Intraspecific variability was detected in *C. tricornis* and *N. drygalskii*. Also, interspecific variability against both bacterial strains from culture collections was detected within the genus *Camptoplites*.

4. Discussion

All bryozoan species tested in this study caused growth inhibition of some bacteria, showing selective antimicrobial activity. This selective antimicrobial activity may allow some bacteria strains to grow (those associated with bryozoans), while other pathogen bacteria are inhibited (Kelman et al., 2001). The activity found against gram-negative and gram-positive bacterial strains demonstrates that the extracts of Antarctic bryozoans could cause inhibition of bacteria with a distinct cell wall composition, as this happens in other invertebrate groups, such as tunicates (Sivaperumal et al., 2010). Moreover, our results suggest that the presence of lipophilic antimicrobial compounds is greater than that of hydrophilic compounds. Conversely, in the case of repellent/cytotoxic compounds, previous studies showed a clear trend

Table 4
Antimicrobial activity of ether extracts of Antarctic bryozoans. Level: weak inhibition (0–1 mm, +), moderate inhibition (>1–3 mm, ++), strong inhibition (>3–7 mm, +++) and very strong inhibition (>7–15 mm, ++++). Blank space = inactive (Lippert et al., 2003).

Species	*B. cereus* CECT 4014	*E. coli* ATCC 700078 WG5	BAC02.2	BAC02.1	BAC84	*P. luti* CECT 5885^T	*S. livingstonensis* CECT 5933^T
Bostrychopora dentata		+	+				
Camptoplites angustus (1)		+	+	+++	++		
Camptoplites angustus (2)		+	+	++			
Camptoplites bicornis	+++	+	+++	++++	++	+++	++
Camptoplites tricornis (1)	++	+		++			
Camptoplites tricornis (2)	++	+	++	+++	+	+++	++
Dakariella dabrowni		++					
Isoschizoporella secunda	++		+	+			
Isosecuriflustra tenuis			++	+++	++		
Klugella echinata	+++	+	+	++			
Melicerita obliqua	+	+	+	+			
Nematoflustra flagellata			+	++			
Notoplites drygalskii (1)		++	+	+++	+		
Notoplites drygalskii (2)	++	++	+	++			
Smittina antarctica	++	+	+	+			
Stystenopora contracta	++		+, ++[a]	+			

[a] Level of the activity of the only butanol extract. These categories, however, have to be considered with caution since the diffusion rate of some antimicrobial compounds could perhaps affect the size of the halii too (Martín-Rodríguez et al., 2014).

towards a combination of lipophilic and hydrophilic compounds acting against sympatric micro and macropredators in Antarctic bryozoans (Figuerola et al., 2013b, 2014).

This study is one of the first attempts to investigate the antimicrobial activity of common and abundant Antarctic bryozoan species against sympatric bacteria (Lebar et al., 2007; Sharp et al., 2007). Most of the Antarctic bacterial strains isolated here are representative of genera associated with bryozoan surfaces (Pukall et al., 2001; Heindl et al., 2012). Thus, this study uses a realistic approach to determine the possible roles of bryozoan metabolites in their habitats. Remarkably, only a few species of bryozoans have been investigated in the frame of chemical ecology (Sharp et al., 2007).

The bryozoan species, tested herein, exhibited antimicrobial activity against a diverse array of Antarctic bacterial strains. During evolution, these bryozoan species could have acquired antimicrobial defenses against these sympatric bacterial strains probably due to their potential negative effects on these sessile organisms. Also, Peters et al. (2003) found that the circumpolar bryozoan species *F. foliacea* exhibited antimicrobial activity, through alkaloids, against one or more marine bacterial strains. Moreover, Lippert et al. (2003) found that some cold-water bryozoans were chemically defended against diverse marine bacterial strains. Consistent with the extended activity recorded here against some bacterial strains, Antarctic sessile organisms are subjected to intense predation pressure, which has led to the development of chemical defenses in a high percentage of species, including bryozoans (Lebar et al., 2007; Avila et al., 2008; McClintock et al., 2010). In fact, Lippert et al. (2003) suggested, for some cold-water bryozoan species, an inverse relationship between antimicrobial activity and the level of fouling observed. In particular, micro-fouling organisms can attract macro-foulers and, consequently, micro and macropredators. Moreover, these foulers may reduce rates in filter feeding by interfering in zooid currents. Thus, antimicrobial activity could be a potential successful strategy against bacteria and, in consequence, a defense against the subsequent macro-foulers and predators. In the case of the extracts studied here, all of them exhibited at least one kind of repellent and/or cytotoxic activity against sympatric micro and/or macropredators (Figuerola et al., 2013b, 2014). Thus, some bryozoan species tested here show diverse types of activity (antimicrobial, repellent and cytotoxic), suggesting that their compounds are used for a wide array of ecological roles. Other studies reported different defensive mechanisms in the same organism. In particular, the Antarctic soft corals *Alcyonium paessleri* and *Germsemia antarctica* possess compounds with repellent, antifouling and toxicity properties (e.g. Slattery and McClintock, 1995, 1997; Slattery et al., 1995). Among other bryozoan species, the lack of one type of repellent/cytotoxic activity could be compensated by the antimicrobial activity and vice versa. Our study confirms previous hypothesis suggesting that these bryozoan species are particularly active against macro and microorganisms. In fact, bryozoan metabolites, mainly comprising polyketides and alkaloids, are responsible for different ecological activities, like antifouling and antipredation (Blunt et al., 2004; Lopanik et al., 2004; Lebar et al., 2007; Sharp et al., 2007; Kornprobst, 2010; Figuerola et al., 2013b, 2014). Moreover, there is also evidence that bryozoan compounds possess cytotoxic compounds. For example, the circumpolar species *F. foliacea*, present alkaloids with cytotoxic activity against the human colon cancer cell line HTC-116 (Lysek et al., 2002), and the Antarctic bryozoan species *Carbasea curva* (Kluge, 1914), is known to produce active compounds with haemolytic properties against erythrocytes derived from different mammals (Winston and Bernheimer, 1986). Thus, our results, together with previous data, suggest the existence of a wide array of potential active bryozoan metabolites.

The lack of (or low) activity recorded in some bryozoan species against certain Antarctic bacterial strains (BAC03, BAC84, *P. luti* and *S. livingstonensis*) might be explained when considering co-evolution, although more bryozoan species have to be tested to prove it. In this sense, bacteria which may often come into close contact with bryozoans may have developed resistance to their metabolites (Lippert et al., 2003). Also, some bryozoan species might not be prone to certain bacterial pathogens or be susceptible to them, as discussed by Koplovitz et al. (2011).

It should also be noticed that some associated bacterial populations might appear as favourable microorganisms, being a source of nutrition for the organism host (Sorokin, 1978). This might be the case of the bacterium isolated from the sympatric sponge (*Haliclona* sp.) tested here. Similarly, previous studies tested antifouling activity of Antarctic sponges and ascidians against bacterial strains isolated from sympatric invertebrates (Peters et al., 2010; Koplovitz et al., 2011). Nonetheless, some bacteria may express pathogenicity only under certain conditions (Kushmaro et al., 1997). Remarkably, there is the possibility that it is only when bacteria become pathogenic, that the bryozoans increase the concentration of defensive metabolites around the diseased areas, thus

regulating symbiotic bacteria (e.g. Lopanik et al., 2006). Hence, testing the antimicrobial activity against bacteria isolated from the same organism may or may not lead to the detection of antibacterial activity, as described for corals (Kelman et al., 1998; Newbold et al., 1999).

Most ether extracts tested here were active against a collection of bacterial strains, suggesting a high potential antibacterial activity and consequently, a high biomedical potential. This is in contrast to the lack or low activity against three of the Antarctic isolates (BAC03, *P. luti* and *S. livingstonensis*). In fact, antimicrobial activity against non-marine pathogens does not necessarily indicate similar activity against marine bacteria (Lippert et al., 2003). Although Colon-Urban et al. (1985) detected a moderate inhibition of growth for standard bacteria *Staphylococcus aureus* Rosenbach 1884, in the crude extract of *N. flagellata*, our results showed a lack of activity for this species against collection bacterial strains. Thus, more tests against a wider array of bacterial strains are needed. In a pharmacological context, our results confirm that marine natural products from bryozoans may be good sources of useful antimicrobial compounds (Tadesse, 2010). However, Tadesse (2010) showed that only some Arctic and sub- Arctic bryozoan species displayed antimicrobial activity against human and fish pathogenic bacteria. The antimicrobial activity against *E. coli* found in our results was previously reported for other bryozoan species, such as the circumpolar *F. foliacea* and *Bugula pacifica* Robertson, 1900 (Shellenberger and Ross, 1998; Peters et al., 2003). Moreover, *F. foliacea* and *B. pacifica* also exhibited antimicrobial activity against *Bacillus subtilis* (Ehrenberg 1835) (Holst et al., 1994; Shellenberger and Ross, 1998). The high frequency of potential antimicrobial activity found in this study might be consequence of the high concentrations of bacteria to which filter feeders, like bryozoans, are exposed and consequent disadvantages, as mentioned above (Shellenberger and Ross, 1998; Sharp et al., 2007; Tadesse, 2010).

Variability of bioactivity within the same species collected from distant geographic locations was also detected in our bioassays. For instance, *N. drygalskii* showed antimicrobial activity against *B. cereus* CECT 4014 and strain BAC84 in only one of the two localities were it was collected. Moreover, *C. angustus* and *C. tricornis* caused different inhibition against some bacteria in the two localities where individuals were collected. Particularly, one sample of *C. tricornis* showed more than twice as much activity as another. It was previously reported that antimicrobial activity could differ within populations of the same species (Lippert et al., 2003). For example, *F. foliacea* from the North Sea produced flutramine E 68 with antimicrobial activity against *B. subtilis* (Holst et al., 1994), while the same species from Canada did not produce this compound, but contained another flustramine (D 67), which had similar antimicrobial activity against the same bacteria (Laycock et al., 1986). Also, chemical variability might also vary with different depths. Indeed, two distinct chemotypes have been found within *Bugula neritina* (Linnaeus, 1758) populations at the same location but at different depth (Davidson and Haygood, 1999). Moreover, considering that Antarctic species show a wide eurybathy (Figuerola et al., 2012a), the variability of their bioactivity could also be related to their potential to live in a wide range of depths. Hence, our findings suggest intraspecific variability in bioactivity depending on location and/or depth, as an adaptive response to diverse abiotic and biotic factors and/or genetic variability, as other authors reported for other marine invertebrates (e.g. Noyer et al., 2011; Pawlik, 2012).

In relation to temporal patterns of compound production, Peters et al. (2004) demonstrated that the concentration of alkaloids in *F. foliacea* fluctuated throughout the year, being another possible cause of intraspecific variability. Furthermore, there is evidence that the origin of some compounds is related to endosymbiotic microorganisms. In fact, it has been reported that some species of *B. neritina* contained chemical compounds resistant to predation (bryostatins) produced by their bacterial symbionts, *Endobugula sertula* Haygood and Davidson 1997 (Lopanik et al., 2006). However, some northern forms of this species did not possess them (McGovern and Hellberg, 2003).

With regard to interspecific variability, the ether extracts of two species of *Camptoplites* (*C. bicornis* and one sample of *C. tricornis* (2)) affected all bacterial strains tested except one (strain BAC03). Additionally, *C. bicornis* displayed stronger inhibition against one collection bacterial strain than any other extract tested. In fact, the strong activity previously recorded in sub-Arctic bryozoans against a variety of different bacterial strains suggested the presence of a broad-spectrum of active compounds (Lippert et al., 2003), being probably the case of some species of *Camptoplites* tested here.

In summary, our study suggests that antimicrobial activity is relatively common among Antarctic bryozoans. Understanding the ecological complex process of chemical interactions between hosts and unicellular and multicellular epibionts is fundamental to understand the functions of antimicrobial metabolites. This is especially relevant in stable ecosystems, such as Antarctica, where biological traits are the main factors structuring benthic communities. In particular, this study is the first step towards the screening and further structural elucidation of potential novel marine compounds for Antarctic bryozoans. Different intra/interespecific patterns of antimicrobial activity were found, suggesting that Antarctic bryozoan species produce diverse bioactive metabolites. In addition, the genus *Camptoplites* inhibited most of the studied bacterial strains, indicating a broad-spectrum of antimicrobial activity. Moreover, the strong antimicrobial activity against collection bacterial strains recorded for some species in this study suggests that further exploration on this topic could lead to the potential use of these extracts as antimicrobial drugs against pathogenic bacteria.

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Quantification of tetracycline and chloramphenicol resistance in digestive tracts of bulls and piglets fed with Toyocerin®, a feed additive containing *Bacillus toyonensis* spores

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ABSTRACT

The complete genome sequencing of *Bacillus toyonensis*, the active ingredient of the feed additive Toyocerin®, has revealed the presence of *tet*M and *cat* genes, a tetracycline and a chloramphenicol resistance gene, respectively. The aim of this study was to determine whether the use of Toyocerin® (viable spores of *B. toyonensis*) as a probiotic in feedstuff increased the abundance of tetracycline and chloramphenicol resistant bacteria in the intestinal tracts of piglets and Holstein bulls. To this end, qPCRs were designed to quantify the abundances of *tet*M and *cat* genes and *B. toyonensis* in the intestinal content of animals treated and non-treated with Toyocerin®. Additionally, the culturable bacterial populations resistant to tetracycline or chloramphenicol were enumerated by plate counting. No statistical significances were detected between the concentrations of tetracycline or chloramphenicol resistant bacterial populations in treated and non-treated animals. The concentrations of *tetM* and *cat* in most of the treated animals were similar to those of *B. toyonensis*. Furthermore, *tetM* and *cat* genes were also detected in some non-treated animals, although in low concentrations. These results suggest that *tet*M and *cat* genes are already circulating among the commensal microbiota regardless of the use of Toyocerin®. The use of Toyocerin® as a supplement in feedstuff does not increase the abundances of tetracycline and chloramphenicol resistant bacteria in the intestinal tracts of piglets and Holstein bulls beyond the contribution directly associated to the introduction of *B. toyonensis* spores through diet.

1. Introduction

Toyocerin® is a commercial preparation used as a feed additive in animal nutrition. Its active ingredient is viable spores of *Bacillus toyonensis* BCT-7112^T, a non-genetically modified, Gram-positive, rod-shaped bacterium originally isolated from soil in Japan. Several studies with *B. toyonensis* have shown beneficial effects in animals, such as improvement in digestive health and weight gain (Kirchgessner et al., 1993; Lodemann et al., 2008; Scharek et al., 2007; Schierack et al., 2007; Taras et al., 2005; Vilà et al., 2009). In parallel, over the last thirty years, a number of toxicological studies have been performed with Toyocerin®, reporting no toxicity in a wide range of doses, routes of exposure, length of administration and types of animals, including healthy humans (Williams et al., 2009).

B. toyonensis BCT-7112^T was initially identified as a strain belonging to *B. cereus*. However, a recent extensive study has provided phenotypic and genotypic evidence indicating that strain *B. toyonensis* BCT-7112^T represents a new species

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within the *Bacillus* genus (Jiménez et al., 2013b). The complete genome of *B. toyonensis* has been sequenced and annotated, shedding light on some interesting genetic characteristics of this probiotic microorganism (Jiménez et al., 2013a). In particular, bioinformatic analyses of the complete genome (chromosome and plasmids) have revealed the presence of *tet*M and *cat*, a tetracycline and a chloramphenicol resistance gene, respectively. The *tet*M gene, which is widely distributed among Gram-positive bacteria, codes for a ribosomal protection protein that reduces the susceptibility of ribosomes to the action of tetracycline, doxycycline and minocycline (Burdett, 1996). The *cat* gene codes for a chloramphenicol-aminotransferase enzyme which catalyzes the acetyl coenzyme A-dependent acetylation of chloramphenicol (Schwarz et al., 2004).

The presence of tetracycline and chloramphenicol resistance genes in *B. toyonensis* raises some concern regarding potential antibiotic resistance transfer from the probiotic bacteria to other bacteria in the intestinal tract, since commensal and pathogenic bacteria may be susceptible to exchanging DNA by conjugation, transduction or transformation mechanisms (Ashraf and Shah, 2011; Klein, 2003; Mathur and Singh, 2005; Salyers et al., 2004; Snydman, 2008). Thus, it is of great importance to ensure that probiotic bacteria intended for human or animal consumption do not transfer any antimicrobial resistance genes in the gastrointestinal tract (European Food Safety Authority, 2012; Gueimonde et al., 2013; Leuschner et al., 2010).

The present study was conducted to determine whether feeding with Toyocerin® (viable spores of *B. toyonensis*) could cause a significant increase of tetracycline and chloramphenicol resistant bacteria already present in the intestinal tracts of piglets and Holstein bulls. To this end, we designed qPCR primers to quantify and compare *tet*M and *cat* genes in animals fed with the feed additive preparation Toyocerin®, against a control group. *B. toyonensis* was also quantified to determine whether the potentially higher concentration of these genes was related to the probiotic supplement. Additionally, we enumerated by plate counting the culturable bacterial populations resistant to tetracycline or chloramphenicol in the intestinal content of animals fed and non-fed with Toyocerin®.

2. Materials and methods

2.1. Bacterial strains and media

B. toyonensis BCT 7112^T, *B. cereus* Rock1-3, *B. cereus* BAG1O-2, *B. cereus* BAG6O-1, *B. cereus* Rock3-29, *B. cereus* VD148, *B. cereus* BAG4X2-1, *B. cereus* BAG5O-1, *B. cereus* Rock3-28, *B. cereus* VD115 were used as reference strains. All of them belong to the same genomospecies, with *B. toyonensis* BCT 7112^T as the type strain (Jiménez et al., 2013b). Additionally, other closely related species were used, namely *B. asahii* JCM 12112^T, *B. cereus* CECT 148^T, *B. mycoides* CECT 4128^T, *B. pseudomycoides* CECT 7065^T and *B. thuringiensis* CECT 197^T.

B. toyonensis strains were grown aerobically at 37 °C in a specifically designed medium containing 20.0 g/L maltodextrin, 15.0 g/L soy peptone, 5.0 g/L yeast extract and 5.0 g/L sodium chloride, adjusted to pH = 7.2 ± 0.1. The other strains were grown in Tryptic Soy Broth (Pronadisa, Barcelona, Spain) at the same temperature. Strains were stored at −80 °C with 15% (vol/vol) glycerol. Fresh cultures were obtained when required by over-night growing at 37 °C in the corresponding medium.

The *B. toyonensis* medium was supplemented with 8.0 μg/L of tetracycline (Sigma, St. Louis, MO, USA) or 8.0 μg/L of chloramphenicol (Sigma, St. Louis, MO, USA), and 15 g/L agar for enumeration of the populations resistant to tetracycline and chloramphenicol, respectively. The antibiotic concentrations were selected following the cut-off values defined by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority to determine antibiotic resistance in susceptible bacteria (European Food Safety Authority, 2012).

2.2. Sample collection

Rumen content samples from bulls and colon content samples from piglets were provided by Rubinum SA from previous studies intended to evaluate the effects of Toyocerin® on ruminal and intestinal parameters (non published). Briefly, 40 Holstein bulls with an average age of 218.6 ± 2.62 days and an initial body weight of 291.9 ± 4.8 kg were distributed in two treatment groups: with or without Toyocerin® feed. Each group was located in a separated barn equipped with a computerized concentrate feeder, a waterer and seven straw feeding spots. The concentration of *B. toyonensis* in feed was 0.2 g/kg, achieving 0.2×10^9 CFU *B. toyonensis*/kg feed. Animals were slaughtered after 90 days of the onset of the study and rumen contents (20 Toyocerin® feed and 20 control) were collected and immediately frozen at −80 °C until use. Similarly, 30 weaned piglets with an average age of 21 days were separated into two treatment groups: with or without Toyocerin® feed. The concentration of *B. toyonensis* in feed was 1 g/kg, achieving 1.0×10^9 CFU *B. toyonensis*/kg feed. Animals were slaughtered after 35 days of the onset of the study and colon contents (15 Toyocerin® feed and 15 control) were collected and immediately frozen at −80 °C until use.

2.3. DNA extraction

DNA from the rumen or colon content was extracted using the Repeated Bead Beating method described elsewhere with some modifications (Yu and Morrison, 2004). Briefly, 250 mg samples of frozen digestive tract content (from piglets or bulls) were transferred into a screw-cap tube containing 1 mL of lysis buffer (500 mM NaCl, 50 mM Tris–HCl pH = 8.0, 50 mM EDTA pH = 8.0 and 4% SDS) and a combination of glass beads (1 piece of 3 mm, 0.25 g of 1 mm and 0.37 g of 0.1–0.11 mm). Samples were homogenized for 30 s at 6.0 m/s using a Fast Prep®-24 Instrument (MP Biomedicals, Santa Ana, CA, California), incubated at 70 °C for 15 min and centrifuged for 5 min at 16,000 × *g*. The supernatant was transferred to a 2 mL microtube. 300 μL of fresh lysis buffer was added to the lysis tube and the procedure repeated. Then, the supernatants from both repetitions were pooled and 260 μL of 10 M ammonium acetate was added, followed by incubation on ice for 5 min. Samples were centrifuged at 16,000 × *g* for 10 min and the supernatant recovered. The DNA was precipitated by adding

one volume of isopropanol and incubating the solution on ice for 30 min followed by washing twice with ice-cold 70% ethanol. Finally, DNA was centrifuged at 16,000 × *g* for 15 min, dried under vacuum for 5 min, and resuspended in 100 μL of 50 nM Tris-EDTA pH = 8.0.

DNA from the type strains of *Bacillus* spp. were extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions for Gram-positive bacteria.

2.4. Development of specific qPCRs

2.4.1. Primers and probe design

Primers were designed to amplify the tetracycline resistance gene *tet*M and the chloramphenicol resistance gene *cat* from *B. toyonensis* and some closely related species (*B. thuringiensis, B. cereus*, *B. weihenstephanensis* and *B. mycoides*). To this end, the primers were designed to target conserved regions of *tet*M and *cat* genes. Sequences of the genes from complete genomes of these species were retrieved from the GenBank database and aligned using the BioEdit program (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) (Table S1). All the strains included in the alignment had an average nucleotide identity (ANI) to *B. toyonensis* higher than 89.5% (Jiménez et al., 2013b). For the selective quantification of *B. toyonensis*, primers and a TaqMan®-probe were designed to amplify a specific region of the *ccpA* gene of the *B. toyonensis* group. Lastly, two universal 16S rRNA gene primers were selected to quantify total bacterial content: 338F and 519R (Mao et al., 2012). After primer design, a BLAST analysis was performed to confirm their specificity for the target genes. The pair primers and probes used for qPCR methods are detailed in Table 1.

2.4.2. Preparation of standard curves

Plasmid constructs were used to generate standards for the qPCR assays. All gene fragments were amplified from a DNA extract of *B. toyonensis* and cloned with pGEM-T Easy vectors (Promega, Madison, WI, USA) following the manufacturer's instructions. The constructs were transformed into *Escherichia coli* JM109 competent cells. The vectors containing the inserts in ampicillin-resistant colonies were purified using the QIagen Plasmid Mini purification kit (Qiagen, Valencia, Spain), and their concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (Thermoscientifics, Wilmington, DE, USA).

To calculate the number of genomic construct copies of each gene stock, the following equation was used: [concentration of pGEM-T Easy::*insert* (ng/μL)/molecular mass (ng/mol)] × 6.022×10^{23} molecules/mol = number of molecules of pGEM-T Easy::*insert*/μL. The stock was 10-fold serially diluted in a 5 ng/μL poly(dI–dC) solution as nonspecific DNA background to prepare the standard curve for qPCR. The standard dilutions were distributed and stored at −80 °C until use. This stock was amplified in triplicate in three independent experiments, and the average of the threshold cycle (C_t) results was used to obtain the standard curve for each gene. Three replicates of each dilution were added to each qPCR mixture in each experiment.

2.4.3. qPCR method

*tet*M, *cat* and 16S rRNA genes were quantified based on the fluorescent dye SYBR® green. Amplification was performed in a 20-μL reaction mixture containing 10 μL SYBR® Select Master Mix (Life Technologies, Waltham, MA, USA), 200 nM of each primer (except for the 16S rRNA gene where 100 nM of each primer was used) and 2 μL of the DNA sample or quantified plasmid DNA. For *tet*M, the thermal-cycler conditions were as follows: an initial setup of 2 min at 50 °C, followed by 2 min at 95 °C, 40 cycles of 15 s of denaturation at 95 °C, 15 s of annealing at 60 °C and 45 s of extension at 72 °C. For the 16S rRNA gene, a single step of 1 min annealing/extension at 63 °C was performed. Product specificity was confirmed by melting curve analysis (55–95 °C, 0.3 °C per read, 30 s hold). The *ccpA* gene (*B. toyonensis* specific) was quantified using a TaqMan® fluorescent probe. The 20-μL reaction mixture contained 10 μL TaqMan Environmental Real-Time PCR Master Mix 2.0 (Life Technologies, Waltham, MA, USA), 200 nM of each primer, 200 nM of TaqMan® probe, 0.4 μg/μL of BSA and 2 μL of the DNA sample or quantified plasmid DNA. The thermal-cycler conditions were as follows: an initial setup of 10 min at 95 °C, 40 cycles of 15 s of denaturation at 95 °C, and 1 min of annealing/extension at 63 °C. All the samples and standards were run in duplicate and three non-template controls were included in every plate. Additionally, extracts from pure cultures of *B. toyonensis*, *B. asahii*, *B. cereus*, *B. mycoides*, *B. pseudomycoides* and *B. thuringiensis* were analyzed to confirm the specificity of the qPCRs.

2.4.4. qPCR of intestinal tract samples

Samples were analyzed per duplicate and both positive and non-amplification controls were run in each plate. DNA extracts were 10-fold diluted to test for the potential presence of inhibition in the samples due to substances co-purified during DNA extraction. Two dilutions were tested

Table 1
Primer pairs and probes used in the qPCRs.

Target	Primer/probe	Sequence 5′–3′	Reference
tetM	tetM-F	CTGGCAAGACGAGTTTGACTGA	This study
	tetM-R	CCACTTCAGCGATAAAATCAGC	
cat	cat-F	CTAATGTTCATGGTCTCTTCCCGAAA	This study
	cat-R	CTAGTCCAAGGTATCCCAGAAATCG	
ccpA (*B. toyonensis* specific)	Toy-F	CCACGCAGCTCAAGATGCT	This study
	Toy-P	FAM-ACTAATGGTACGCCCGCAG-MGB	
	Toy-R	GCATTGCTACTGCACCGATATC	
16S rRNA	338F	ACTCCTACGGGAGGCAGC	Mao et al. (2012)
	519R	GTATTACCGCGGCKGCTG	

for each sample: direct extract and 1:10 for *tet*M, *cat* and *ccp*A; and 1:10 and 1:100 for 16S rRNA. The C_T was defined as the average of the duplicate data obtained. C_T data were expressed as the number of GCs according to the values obtained with the standard curve generated for each qPCR. Data analysis was carried out with StepOne Software version 2.2.2 (Applied Biosystems, Waltham, MA, USA).

2.5. Quantification of bacterial populations resistant to tetracycline or chloramphenicol

Samples of 500 mg of digestive tract content (either rumen or colon, from Holstein bulls or piglets, respectively) were transferred into a tube containing 4.5 mL of Ringer ¼ buffer and thoroughly homogenized by vortexing for 15 min. The mixture was serially 10-fold diluted in Ringer ¼ and at least three dilutions were analyzed in duplicate for each sample. Aliquots of 100 μL were plated on the *B. toyonensis* medium supplemented with the target antibiotic and incubated at 37 °C for 48 h in a humid chamber to prevent plate dehydration.

2.6. Data analysis

Genomic copies and colony forming units were log-transformed as required to normalize the distributions prior to statistical analysis. The student's *t*-test was used to compare average concentrations in animals fed and non-fed with Toyocerin®. When differences in the standard deviations of the compared samples were found to be statistically significant, a comparison was performed using the Mann–Whitney test with the median concentrations. All statistical analyses were performed using the software GraphPad Prim (version 6.03) (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Primer and probe design

The *in silico* analysis showed that the primers designed for *tet*M, *cat* and 16S rRNA hybridized with the corresponding genes from *B. toyonensis* genomospecies and all the strains contained in Table S1, with the exception of *B. cereus* CECT 148^T, which lacked the *cat* gene. The primers and the TaqMan®-probe for *ccp*A gene only hybridized with *B. toyonensis* genomospecies. The ability of the primers to amplify all the targeted species in the present study was additionally verified by running a qPCR using approximately 200 ng of genomic DNA isolated from the reference strains. All the targeted species (*B. toyonensis* genomospecies, *B. cereus, B. mycoides* and *B. thuringiensis*) showed C_t values of between 9 and 14 for *tet*M and *cat* qPCRs, compared with *B. asahii* and *B. pseudomycoides*, which were detected but only at higher C_T values ($C_T \geq 30$). The performance of the universal 16S rRNA gene primers, 338F and 519R, has already been demonstrated in previous studies (Mao et al., 2012). For all the above qPCRs, the absence of nonspecific products or primer dimers was confirmed by the observation of a single melting peak.

When testing the specificity of the *B. toyonensis* specific qPCR based on *ccp*A gene, *B. cereus*, *B. mycoides B. pseudomycoides* and *B. thuringiensis* showed negative C_T values. *B. asahii* was detected, but only at C_T values around the limit of detection (C_t = 39.2), compared with the C_T value of *B. toyonensis* species (C_T average = 20.2). These findings indicate that this qPCR was specific and that a minimum of 200 ng of DNA is required to generate a positive signal for other non-target genetically close microorganisms.

3.2. qPCR standards

Four standard curves were plotted (Fig. 1), one for each qPCR assay targeting one of the genes studied (*tet*M, *cat, ccp*A and 16S rRNA). The standard curves presented are the averages of three independent experiments. The size of the amplified fragments from each gene, the slope and efficiency of the curves generated and their limits of linear quantification are detailed in Table 2.

3.3. Occurrence and quantification of tetM, cat, B. toyonensis and 16S rRNA genes in digestive tract samples

Absolute gene copy numbers of the antibiotic resistance genes (*tet*M and *cat*) and *B. toyonensis* quantified by qPCR are presented in Fig. 2 and Table S2. The absolute number of all genes was normalized to that of the 16S rRNA gene at the respective sample to minimize the variance caused by the differences in existing background bacterial abundances. The concentrations of *tet*M and *cat* genes in the normalized data followed the same trends when compared to those observed in the absolute data, since the 16S rRNA genomic copies were similar among the different samples for each animal (around 10^9 copies/mg in piglets and 10^8 copies/mg in bulls) (Table S2). In addition, no statistically significant differences were observed between the concentration of 16S RNA gene of piglets and bulls fed with or without Toyocerin®.

In piglets non-fed with Toyocerin®, *B. toyonensis* was not detected in any sample, *tet*M was quantifiable only in one, but occurred under the limit of quantification in six

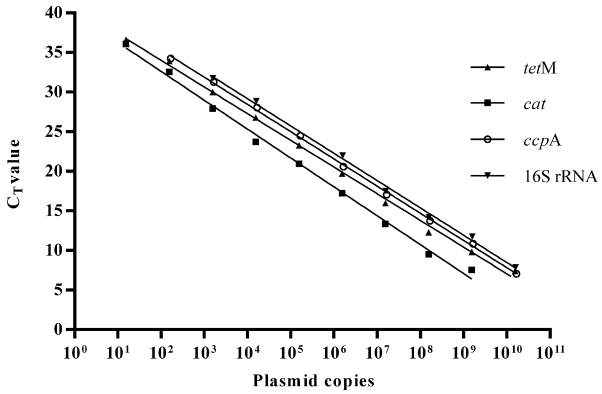

Fig. 1. qPCR standard curves prepared with recombinant plasmids as described above for *tet*M, *cat*, *ccp*A and 16S rRNA genes. The data presented are the averages of three replicates in three independent experiments.

Table 2
Parameters of the qPCR standard curves.

Target gene	Length (base pairs)	Slope and standard deviation	Efficiency of the reaction (%)	Limit of quantification (GC/mg)[a]
*tet*M	216	-3.37 ± 0.03	98.0	5.0
cat	75	-3.65 ± 0.03	88.0	3.5
*ccp*A	143	-3.44 ± 0.01	95.5	33.5
16S rRNA	200	-3.45 ± 0.02	95.0	5.49×10^2

[a] Genomic copies.

more samples, and *cat* was present in all samples. In contrast, *B.* toyonensis, *tet*M and cat were found in all the samples from piglets fed with Toyocerin® (Table S2). The average relative levels of *tet*M and *cat* genes were significantly higher than in samples from animals non-fed with Toyocerin® ($p < 0.0001$). In addition, the concentration of *tet*M genes was similar to that of *B. toyonensis* ($p = 0.0659$).

Regarding the Holstein bulls, no *B. toyonensis* was detected in any of the rumen samples from animals non-fed with Toyocerin®, but was found in 15 out of 20 samples from the ones fed with Toyocerin®, although quantification was only possible in 4 of those samples. The *tet*M gene was unevenly detected in samples at low levels both in animals fed and non-fed with Toyocerin®. *cat* was found in concentrations under the limit of quantification in 10 samples from animals fed without Toyocerin®, but was present at higher concentrations in all samples except one from animals fed with Toyocerin®.

3.4. Enumeration of culturable bacterial populations resistant to tetracycline or chloramphenicol

The results of the enumeration of culturable bacterial populations resistant to tetracycline and chloramphenicol in colon contents from piglets and in rumen contents from Holstein bulls fed with or without Toyocerin® are summarized in Table 3. High levels for both bacterial populations were found in both studied animals in all of the studied groups (fed and non-fed with Toyocerin®). No statistically significant differences were detected between the concentrations of tetracycline or chloramphenicol resistant bacterial populations in animals fed and non-fed with Toyocerin® ($p > 0.05$).

4. Discussion

Probiotic bacteria have emerged as an alternative to increase animal health and promote growth and productivity without the need for antibiotics (Chaucheyras-Durand and Durand, 2010). However, the potential transmission of antibiotic resistance genes from probiotic bacteria to the commensal microbiota in the gastrointestinal tract of animals raises some concern, since there is evidence that intestinal bacteria may interact with transient bacteria as well as serve as a reservoir for antibiotic resistances (Gueimonde et al., 2013). Consequently, the risk that pathogenic and opportunistic microorganisms may acquire new antibiotic resistances and become a threat to animals and humans should not be ruled out (Mølbak, 2004; Moubareck et al., 2003). In this study, we investigated the effects of the feed additive Toyocerin®, a preparation containing viable *B. toyonensis* spores, on the intestinal concentration of tetracycline and chloramphenicol resistant bacteria in piglets and Holstein bulls.

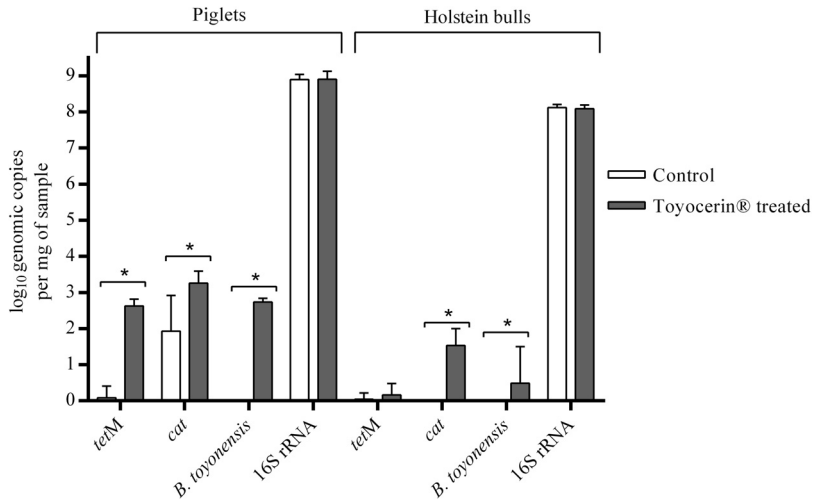

Fig. 2. Absolute concentration of *tet*M, *cat* and 16S rRNA genes, and *B. toyonensis* (*ccp*A gene) in the digestive tracts of piglets and Holstein bulls fed with or without Toyocerin®. Piglets: $n = 30$; 15 treated and 15 non-treated. Holstein bulls: $n = 40$; 20 treated and 20 non-treated. *p-Value < 0.0001.

Table 3
Average of concentration in $\log_{10}$ units (CFU/mg) of culturable bacterial populations resistant to tetracycline or chloramphenicol in the digestive content of animals fed with or without Toyocerin®. Piglets: $n = 30$; 15 treated and 15 non-treated. Holstein bulls: $n = 40$; 20 treated and 20 non-treated.

	Piglets		Holstein bulls	
	Tetracycline	Chloramphenicol	Tetracycline	Chloramphenicol
Toyocerin® treated	6.11 ± 0.38	6.16 ± 0.38	3.89 ± 0.23	4.10 ± 0.25
Control	6.30 ± 0.91	5.99 ± 0.95	3.68 ± 0.70	4.25 ± 0.42

The results from the first approach revealed that the use of Toyocerin® in feedstuff did not significantly increase the concentration of populations resistant to tetracycline or chloramphenicol in the intestinal content of piglets or Holstein bulls. These results are in agreement with the enumerations reported elsewhere for tetracycline in cattle and swine (Stanton et al., 2011; Yang et al., 2010). The second approach showed that the concentrations of *tet*M and *cat* genes in piglets and bulls increased with the addition of Toyocerin® to their diet, as shown in Fig. 2. Interestingly, the concentrations of *tetM* and *cat* in most of the samples were similar to that of *B. toyonensis*, which indicates that the increase observed is directly linked to the introduction of *B. toyonensis* cells or spores into the intestinal tract of the animals through diet. Both genes are located in a structurally conserved region of the chromosome (*tet*M is located in the position 307,023 and *cat* in the position 4,933,576) and neither of them is flanked by mobile genetic elements nor by any other possible horizontal gene transfer mechanism (accession number of the chromosome is CP006863; and accession numbers for plasmids pBCT77 and pBCT8 are CP006864 and CP006865, respectively). As a result, the transference of the gene to other commensal microorganisms seems unlikely. Nonetheless, the potential transference at undetectable low frequencies cannot be completely ruled out.

Furthermore, *tetM* and *cat* genes were detected in some animals non-fed with Toyocerin®, although in low concentrations. The fact that *B. toyonensis* could not be detected in all of these samples suggests that other *Bacillus* sp. strains containing *tet*M or *cat* genes might naturally occur in the intestinal tracts of these animals. Thus, the use of Toyocerin® as a feed additive does not introduce new antibiotic resistance genes into the gut microbiome. In addition, the enumeration by plate counting of the bacterial populations resistant to tetracycline and chloramphenicol in animals fed with Toyocerin® showed that concentrations were up to 2 $\log_{10}$ units higher than those detected by qPCR of the *tet*M or *cat* genes. Consequently, the *tet*M and *cat* genes were only responsible for a small fraction of the phenotypic resistance observed against these antibiotics. Other resistance mechanisms such as tetracycline efflux pumps, ribosomal protection genes, ribosomal structure modifications or the presence of plasmids containing resistance cassettes to tetracycline and chloramphenicol might be naturally present in other commensal bacteria and would explain most of the phenotypic resistance observed in plate counting (Chopra and Roberts, 2001; Galopin et al., 2009; Gueimonde et al., 2013; Lyont and Skurray, 1987).

In summary, the results obtained in this study show that the use of the feed additive Toyocerin® does not increase the existing levels of tetracycline and chloramphenicol resistant bacteria in the intestinal tracts of piglets and Holstein bulls beyond the contribution directly associated to the introduction of *B. toyonensis* spores through diet.

Conflict of interest

This research was funded by Rubinum, S.A. The funders had no role in the study design, data collection, analysis and interpretation or decision to publish the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.07.005.

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Environmental detection and isolation of strains affiliated to *Bacillus toyonensis*, a species with probiotic representants

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Running tittle: *Bacillus toyonensis* in the environment

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Abstract

Bacillus toyonensis (BCT-7112^T), a recently validated *Bacillus* species belonging to the *B. cereus* group, was isolated from soil in Japan in 1966 and it has shown probiotic effects when used as animal feed. The aim of this study was the optimization of a methodology for the detection and isolation of *B. toyonensis* strains to further analyze their occurrence in the environment. A total of 20 samples (soil and sewage sludge) and a collection of *Bacillus* strains isolated from water were screened for the presence of *B. toyonensis* by qPCR. A colony hybridization method targeting the *rpoB* gene was used to isolate potential *B. toyonensis* strains. A total of four *B. toyonensis* strains were isolated from sludge and water. These strains were phenotypically and genotypically characterized using API 50CHB and RAPD, showing some phenotypic and genotypic variability. The proposed methodology showed to be feasible and practical for the isolation of *B. toyonensis* strains from the environment. The developed screening method is a powerful methodology for isolating new *B. toyonensis* strains allowing to demonstrate the ubiquity of this species in different geographical regions and environments with different properties, and contributing to consolidate the genotypic and phenotypic characteristics of this species.

Keywords: *Bacillus toyonensis*; detection; probiotics; sludge; water

Introduction

The benefits of probiotics in the establishment and maintenance of a healthy intestinal microbiota as well as the effect in the immune system stimulation is well documented [1, 2]. Noticeably, in the last decades the use and search of novel probiotic strains for animal husbandry has gained additional interest after the ban on the use of antibiotics as prophylactic agents within the European Union.

Many important probiotic strains used in animal nutrition belong to the *Bacillus* genus due to some of their intrinsic features. *Bacillus* species are Gram-positive, spore forming bacteria, which are capable to resist the harsh conditions posed by the gastrointestinal barrier and allow their easy administration as supplement in the feed. Although there are some important pathogens inside this genus such as some *B. cereus* strains, some species have been shown to provide important beneficial effects in the host and improve the gut health by preventing many intestinal illnesses, since they are able to inhibit the growth of other undesirable intestinal bacteria [3].

The strain BCT-7112^T was isolated from soil in Shizouka Prefecture (Japan) in 1966 and it has been designated as the type strain of *Bacillus toyonesis* (NCIMB 14858^T), recently classified as a new species of the *Bacillus cereus* group [4]. For over 30 years, the spores of *B. toyonensis* have been used in animal nutrition to feed swine, bovine, rabbit, poultry and aquiculture under the commercial brand Toyocerin®. Several studies have shown benefits in health and significant improvements in zootechnical parameters when using this probiotic strain in the feeding of livestock [5-10] . The safety of this strain has been proven by a number of studies carried out *in vitro* and *in vivo* [11-13] as well as its long history of use in animal nutrition worldwide without any report of safety concern. However, there are still some constraints for its acceptance as feed additive in the EU [14]. Therefore, understanding the distribution in the environment of this species would help to improve the knowledge of ecological aspects and its ubiquity in different ecosystems. Consequently, the detection and isolation of environmental *B. toyonensis* strains would be of interest to support scientific opinions and to consider novel strains for use in animal nutrition. At present, a low number of strains belonging to this recently validated new species [15] have been isolated from different geographical sites [4].

The different species belonging to the *Bacillus cereus* group are closely related and many efforts have been made trying to distinguish them [16], especially pathogenic from probiotic strains. For this reason, several molecular detection techniques have been developed like multiplex PCR [17], pulsed-field gel electrophoresis and multilocus sequence typing [18], Fourier transform infrared spectroscopy (FT-IR) [19] and qPCR [20], the last one specifically developed for the detection of *B. toyonensis*.

The aim of this study was the development of a methodology for the detection and isolation of *B. toyonensis* strains from environmental samples to assess their prevalence in the environment but also to be used for the isolation of novel potential probiotic strains. The isolation of *B. toyonensis* is therefore interesting for both its probiotic condition as well as its recent reclassification. These reasons justify the research of new specific molecular detection methods which can be used in environmental samples.

Materials and methods

Strains and growth media

B. toyonensis BCT-7112^T strain (Japanese isolate) and *B. toyonensis* Rock 1-3 strain (North American isolate) were used as positive control in all the experiments, whereas *B. cereus* CECT 148^T, *B. asahii* MA001, *B. pseudomycoides* CECT 7065^T, *B. thuringiensis* CECT 197^T, *B. mycoides* CECT 4128^T were used as negative controls. All the strains were grown in tryptic soy broth (TSB) or agar (TSA) at 30ºC overnight unless otherwise stated.

Samples

A total of 20 environmental samples from soil rich in organic matter (n = 14), and sewage sludge (n = 6) from different wastewater treatment plants were screened for the presence of *B. toyonensis* using the methodology described below. Additionally, a collection of 49 *Bacillus* spp strains previously isolated from drinking water were also screened for the presence of *B. toyonensis*. These strains had been isolated in a previous study, and had been identified as *Bacillus* spp after 16S rRNA gene sequencing.

Sample treatment

The samples were diluted (1:10) in phosphate buffered saline (PBS) and homogenized in a horizontal shaker for 30 min. The supernatant was recovered by decantation and was incubated at 80ºC for 15' to remove the vegetative cells. Finally, 1 ml of the supernatant was inoculated into 9 mL *B. toyonensis* medium containing 20.0 g l^{-1} maltodextrin, 15.0 g l^{-1} soy peptone, 5.0 g l^{-1} yeast extract and 5.0 g l^{-1} NaCl, adjusted to pH=7.2 ± 0.1, and was incubated under agitation and aerobic conditions overnight at 30 ºC.

qPCR amplification

DNA was extracted using standard procedures and the presence of *B. toyonensis* was analyzed using a qPCR targeting the *ccpA* gene (*B. toyonensis* specific) using a specific TaqMan® fluorescent probe as previously reported [20]. The primers and probe used are listed in Table 1.

Isolation of B. toyonensis strains

Samples showing positive amplification by qPCR were further screened by colony hybridization targeting the *rpo*B gene using a dig-labelled probe (Table 1). Briefly, the samples previously enriched in spore forming bacteria as described above, were 10-fold serially diluted in PBS and spread on to TSA agar plates containing or not tetracycline (8 µg ml^{-1}) and chloramphenicol (8 µg ml^{-1}), since some *B. toyonensis* isolates are resistant to these antibiotics, and incubated overnight at 30 ºC. Bacteria were transferred onto Hybond N+ membrane positive charged (Amersham, Pharmacia Biotech, Buckinghamshire, United Kingdom). Bacterial cell lysis was performed by incubation of the membrane onto saturated Whatman paper in the presence of chloroform vapors for 30 min, followed by SDS 10% (W/V) treatment for 5 min, denaturation for 5 min (NaOH 0.5 mol l^{-1}; 1.5 mol l^{-1} NaCl) at RT, followed by incubation at 80 ºC for additional 5 min, neutralization for 5 min (Tris 1M, 1.5 M NaCl, pH: 7.4), followed by a 5 min incubation onto 2x SSC. The membranes were UV irradiated and rinsed with 3x SSC 0.1% SDS for 1 h at 68 ºC. The membranes were pre-hybridized for 1 h at 64 ºC and the probe was finally added and the membranes incubated overnight at the same temperature. Detection of the hybridization was performed using alkaline phosphatase conjugated anti-Dig antibodies with NBT/BCIP substrate (Roche diagnostics, Mannheim, Germany) as described by the manufacturer using standard procedures.

Genotypic characterization

The genetic relatedness among the different isolates was assessed by random amplification of polymorphic DNA, using the primers listed in Table 1. The PCR was performed using Dream Taq Green Master Mix (Life Technologies, Barcelona, Spain) in a final volume of 25 µl containing 1 µmol l^{1} of each primer. The cycling conditions were as follows: initial step at 94 ºC for 5 min, 4 cycles of 94ºC for 5 min, 40ºC for 5 min, 70ºC for 5 min, and 30 cycles at 94ºC for 1 min, 55ºC, for 1 min, 70ºC for 2 min, and a final extension at 70ºC for 5 min [4].

Additionally, the detection of an intergenic region between *ger*IC and *nuc*B that accounts for the presence of the plasmid pBCT77in *Bacillus toyonensis* BCT-7112^{T} was assessed using the primers listed in Table 1. The following cycling conditions: 2 min at 94°C, 30

cycles of 30 s of denaturation at 94°C, 1 min of annealing at 55°C and 3 min of extension at 72°C, followed by a single step of 7 min final elongation at 72°C.

Sequencing

The *ccp*A and 16S rRNA genes were amplified and sequenced using the Big Dye 3.1 terminator kit and an ABI PRISM 3700 DNA Sequencer (Life Technologies, Barcelona, Spain), following the manufacturer's instructions. Similarity search of the sequences was performed using BLAST [21].

Phenotypic characterization

Phenotypic characterization of the isolated strains was performed by commercial API50CHB gallery following the manufacturer's instructions (Biomérieux, Marcy l'Étoile, France). Additionally, the strains were tested for the resistance to tetracycline and chloramphenicol in TSA containing 8 µg/ml of both antibiotics after overnight incubation at 30 ºC which has been associated to the intrinsic *tet*M and *cat*Q genes in this species [22].

Results and discussion

The developed methodology for isolation of *B. toyonensis* consisted in a first enrichment culture in spore forming bacteria, followed by a screening of samples by qPCR targeting the *ccp*A gene [20]. All the analyzed sludge samples (n=6) were positive by qPCR (five samples were from different wastewater treatment plants (WTP) located in Catalonia (Spain) and one sample was from a WTP located in Verona (Italy)). In the case of soil samples no positive results were obtained and they were discarded for further analyses.

Positive samples were screened by colony hybridization targeting the *rpo*B gene. The main difficulty of the methodology was the efficient lysis of the thick cellular wall of this *Bacillus* that was successful after incubation of the colonies in the presence of chloroform vapors. Two isolates were isolated from two different sludges, both from WTPs in Catalonia, namely H3 and 229. The isolates were screened by qPCR targeting the *ccp*A gene and the *ccpA* amplimer was sequenced. The presence of a sequence matching the internal probe used in the qPCR was confirmed in the amplimer for both isolates. In the case of isolate H3, the 16S rRNA gene showed a maximum identity of 100% with *B. toyonensis* BCT-7112^T [NR 121761.1]. In the case of isolate 229, the maximum identity of this gene was 100% with *B. thuringiensis* B407 [NR 102506.1], followed by 99% (1 bp difference (T/C)) with *B. toyonensis* BCT-7112^T [NR 121761.1]. However, the

sequence was identical in this position to other *B. toyonensis* rRNA gene sequences present in the nucleic acid database from GenBank with accession numbers [KP406731.1, KP407120.1, KP407100.1, KP407099.1 and KP407092.1] that correspond to different *B. toyonensis* strains isolated in South Africa and Portugal [23]. Similarly, the *B. toyonensis* Rock 1-3 strain shows and identity of 99% with both the *B. toyonensis* strain BCT-7112^T [NR 121761.1] and *B. thuringiensis* B407 [NR 102506.1] as well as other isolates from a wastewater treatment plant in China [24] . It is remarkable that both *B. toyonensis* strains (*B. toyonensis* BCT-7112^T and *B. toyonensis* Rock 1-3 strain) were originally isolated from soils several decades ago in Japan and North America, respectively, and have been isolated from drinking water and sewage sludge in the Mediterranean region.

On the other hand, two isolates from a *Bacillus* spp. culture collection, namely T 4.2 and T 14.2, previously isolated from drinking water were screened and included in the study due to the high nucleotide identity of the *ccpA* and 16S rRNA genes with *B. toyonensis* BCT-7112^T genes. In both isolates the *ccpA* gene and 16S rRNA gene was identical to that one of the 229 isolate.

Genomic relatedness between the different strains was analyzed by RAPD analysis using 3 different primers. As shown in Fig.1, the H3 isolate (lane 2) showed the same DNA fingerprint as the *B. toyonensis* BCT-7112^T (lane 1) for all the 3 primers used, while the 229, T 4.2 and T 14.2 isolates (lanes 3, 5 and 6) showed a more heterogeneous fingerprint depending on the primer used. Although for RAPD 1 these isolates had a different DNA fingerprint, the RAPD 2 profile was very similar between these 3 isolates together with the profile of the *B. toyonensis* Rock 1-3 strain, although the assay was limited by the low number of bands that amplified for these isolates. In the RAPD3, the T 4.2 and T 14.2 showed a very similar fingerprint, while the H3 and 229 isolates were grouped together with the *B. toyonensis* type and Rock 1-3 strains.

Concerning the phenotypic characterization using API50CHB gallery (Table 2), again there was some diversity in the fermentation pattern of different substrates between the different isolates, although again the fermentation pattern of the H3 isolate was nearly identical with the *B. toyonensis* BCT-7112^T, while the fermentation pattern of isolates 229, T 4.2 and T 14.2 were closer to the *B. toyonensis* Rock 1-3 strain, with few exceptions such as fermentation of D-mannose and gentiobiose in isolate T 4.2.

Antibiotic resistance to chloramphenicol and tetracycline, which has been previously reported in *B. toyonensis* BCT-7112^T [20] was also detected in the H3 isolate, whereas the other isolates were sensitive to both antibiotics, including the *B. toyonensis* Rock 1-3 strain. Additionally, the presence of an intergenic region between *ger*IC and *nuc*B genes, which has previously been linked with the presence of plasmid pBCT77 and suggested to

be related to some unknown complementary mechanisms to resistance to both antibiotics in this *Bacillus* species [14, 25] was assessed. The intergenic region *gerIC-nucB* was only detected in the H3 isolate. The absence of this intergenic region in the above mentioned isolates belonging to the same taxonomic unit of *B. toyonensis* is supporting the difference in the phenotypic resistance to tetracycline and chloramphenicol, which was already reported as intrinsic resistance to the species and related to the chromosomic *tetM* and *catQ* genes [22]. Accordingly, these new strains of *B. toyonensis* could be good candidates to assess their probiotic effects in animal nutrition.

In conclusion, the developed screening method is a powerful methodology for isolating new *B. toyonensis* strains allowing to demonstrate the ubiquity of this species in different geographical regions and environments with different properties, and contributing to consolidate the genotypic and phenotypic characteristics of this species.

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Tables and Figures

Table 1. Probes and primers used in this study

Primer name	Target	Sequence 5'-3'	References
ccpA-Probe	*ccp*A	P FAM-ACTAATGGTACGCCCGCAG-MGB	[20]
Toy-F		CCACGCAGCTCAAGATGCT	
Toy-R		GCATTGCTACTGCACCGATATC	
27F	16S rRNA	AGAGTTTGATCMTGGCTCAG	[27]
1492R		TACGGYTACCTTGTTACGACTT	
LAC2	RAPD1	TGC GCA ACT GTT GGG AAG GG	[26]
241SEQ7+	RAPD2	CGA GCT TCG CGT ACC ACC CC	
241SEQ5+	RAPD3	CGC TGC GGT TGC GCG CCG CC	
rpoB-probe	*rpoB*	TGTAAAAGCTCTCCTACTGAACGTAAACGACGGT	This study
IG_A_F	Intergenic region *gerIC - nucB*	GCCCCTTTAAACTTCATTATGC	This study
IG_A_R		TCCATCCAATCACAAACCAA	
IG_B_F		CCGAGAAGTGCAAAGTAG	
IG_B_R		CCCTTACCTTCAAGTATTCC	

Table 2. Phenotypic characterization of the isolated strains with respect to the probiotic strain *B. toyonensis* BCT-7112^{T}and *B. toyonensis* Rock 1-3 by API 50CHB. Only tests showing positive fermentation are shown (+, positive fermentation; w, weak fermentation).

test	Isolate H3	Isolate 229	Isolate T 4.2	Isolate T 14.2	*B. toyonensis* BCT-7112^T	*B. toyonensis* Rock 1-3
GLY	w				w	
RIB	w	w	w	w	+	w
GAL	w				w	
GLU	+	+	+	+	+	+
FRU	+	+	+	+	+	+
MNE			+			
MDM	w				w	
MDG	+				+	
NAG	+	+	+	w	+	+
AMY	w	w	+		w	w
ARB	+	+	+	+	+	+
ESC	+	+	+	+	+	+
SAL	+	+	+	+	+	+
CEL	w	w	+	w	w	w
MAL	+	+	+	+	+	+
MEL	w				w	
SAC	+				+	
TRE	+	+	+	+	+	+
AMD	+	+	+	+	+	+
GLYG	+	+	+	+	+	+
XLT						w
GEN			+			w
TUR	+				+	
GNT		w	w		w	w

GLY, glycerol; RIB, D-ribose; GAL, D-galactose; GLU, D-glucose; FRU, D-Fructosa; MNE, D-mannose; MDM, methyl-□D-mannopyranoside; MDG, methyl-□D-glucopyranoside; NAG, N- acetylglucosamine; AMY, amygdaline; ARB, arbutine; ESC, esculine; SAL, salicine; CEL, D-cellobiose; MAL, D-maltose; MEL, D-melibiose; SAC, D-saccharose; TRE, D-trehalose

AMD, amidon; GLYG, glycogen; XLT, xylitol; GEN, gentiobiose; TUR, D-turanose; GNT, potassium gluconate

Fig. 1. Genetic relatedness among the different isolates and control strains analyzed by Random Amplification of Polymorphic DNA (RAPD): a) RAPD 1; b) RAPD 2; c) RAPD 3. M, Gene ruler 100 bp plus DNA ladder (Life Technologies); 1, *B. toyonensis* BCT-7112T; 2, isolate H3; 3, isolate 229; 4, *B. toyonensis* Rock 1-3; 5, isolate T 4.2; 6, isolate T 14.2; 7, negative control.

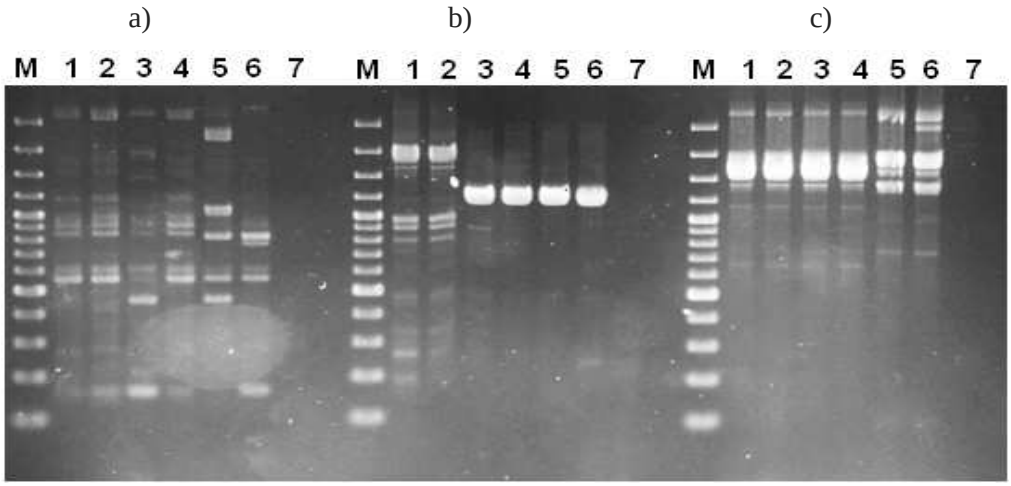

Fig. 2. Amplification of the intergenic region *gerIC-nucB* in the isolated strains: IG_A (amplimer 1830 bp), IG_B (amplimer 852 bp). M, Gene ruler 100 bp plus DNA ladder (Life Technologies); 1, isolate T 4.2; 2, isolate H3; 3, isolate 229; 4, *B. toyonensis* BCT-7112T; 5, *B. toyonensis* Rock 1-3; 6, isolate T 14.2; 7, negative control.